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13. ABSTRACT ***(Maximum 200 Words)***

There is strong evidence that the expression of genes involved in signal transduction such as protein kinases is altered in tumor cells and that the aberrant expression of one or several of these genes parallels the progression of tumors to a more malignant phenotype. We developed a DNA micro-array based screening system to monitor the level of expression of tyrosine kinase (tk) genes and to derive quantitative information to support prognostication and therapeutic decisions. In the first year of this effort, we finished the development and testing of hardware necessary to prepare the DNA micro-arrays, performed RNA extraction, cDNA preparation and labelling reactions, and defined a working protocol for array hybridization and quantitative analysis. Our prototye design simultaneously measures the expression of 63 different tk genes. Additional targets for investigation will be defined by the ongoing molecular cloning and sequencing of gene transcripts found in prostate cancer cell lines and clinical specimens. Using a set of phenotypically well characterized prostate cancer cell lines, the system has proven to be able to deliver reproducible data regarding changes in tk gene expression during cell transformation and progression towards a more malignant phenotype.

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Table of Contents

INTRODUCTION:

Aberrant expression of receptor or cytosolic tyrosine kinase (tk) genes and, in particular, their hyper-expression are common phenomena in prostate cancer, which are believed to alter cell growth and response to external signals such as growth factors, hormones etc. Knowledge about the relative levels of expression of many tyrosine kinase genes, all at the same time, might contribute significantly to a better understanding of the processes of tumor development and progression. We are developing a rapid assay that will use innovative cDNA micro-arrays carrying small amounts of individual tyrosine kinase gene-specific targets to simultaneously determine the expression levels of up to 100 tyrosine kinase genes using a small number of cells. Three years of research and development are expected to lead to discovery of a set of gene-specific markers associated with prostate cancer progression and a simple device capable of performing inexpensive expression profiling of these markers. The research and development efforts in the first year of this 3-year project focused on the design and testing of robotic instruments to prepare DNA micro-arrays and the preparation of prototype arrays carrying sets of more than 50 gene-specific tyrosine kinase fragments. The second year effort (i.e., the reporting period 01 Mar 2001 – 28 Feb 2002) was directed towards the optimization of cDNA preparation, labeling, hybridization and detection protocols as well as the molecular cloning and sequencing of prostate-specific tyrosine kinase gene transcripts.

BODY: The following summarizes our progress as it relates to the approved 'Statement of Work'.

Task 1. Identify tyrosine kinase (tk) genes expressed in normal and neoplastic breast tissues

1.1 Prepare cDNAs from six cell lines and ten frozen tissue specimens

We isolated total RNA from 15 different prostate cell lines (Table I) and ten frozen tissue specimens. Exponentially growing cell lines were provided to us by various collaborators, while all ten prostatic tumor tissues were obtained from the Cancer Tissue Core facility at the Comprehensive Cancer Center, University of California, San Francisco (UCSF)(B.M. Ljung, M.D. and K. Chew). The actual number of cell lines processed is higher, because we received aliquots of LNCaP cells from Drs. S. Hayward (Vanderbilt University) and Ron Jensen (UCSF).

Table I. Prostate specific cell lines used in our experiments.

DU145	ND-1
PC-3	LNCaP
BPH-1 CAFTD-01	BPH-1 CAFTD-02
BPH-1 CAFTD-03	BPH-1 CAFTD-04
BPH-1 CAFTD-05	BPH-1 CAFTD-06
BPH-1 CAFTD-07	BPH-1 CAFTD-08
BPH-1 TETD-A	BPH-1 TETD-B
BPH-1	

We received a total of 10 frozen tissue specimens representing two normal tissue specimens and eight prostate cancer tissues. The RNA was extracted from these tissues using a commercial kit (Qiagen) and transcribed into cDNA immediately after isolation using a second kit (Ambion). Remaining RNA was stored at –80°C. We prepared cDNAs from the various RNA samples by random priming and reverse transcription. Commercial kits (Qiagen, Roche, Ambion) were used for all steps. Typically, 1 μg of total RNA produced sufficient quantities of cDNA for gel electrophoritic quality control, cloning and/or repeated micro-array analyses. Table I lists the names of the 15 cell lines used in our studies. In addition, Dr. Hayward provided 11 tissue samples of BPH-1 derived tumors grown in nude mice.

1.2 Perform RT-PCR reactions and clone PCR products in plasmids

We used about 100 ng of cDNA in PCR reactions to amplify tk-specific cDNA fragments with our mixed-base F-/R-TYRK primers. PCR amplification was performed for 35 cycles using mixed base primers that bind to the conserved sequences of domains VII and IX of the tyrosine kinase genes (F-TYRK: 5′-GGGCGTCAGAARRTNRSNGAYTTYGG-3′; R-TYRK: 5′-GCGCGGGCCCRWANSH-CCANACRTCNSA-3′). Each cycle consisted of a denaturation step of 30 sec at 94°C, primer annealing at 53°C for 60 sec and primer extension for 120 sec at 72°C. Most PCR products appeared as a single broad band of the expected size (~160-170 bp). The amplification products were separated on a 4% agarose gel, and a slice containing fragments of approximately 160-180 bp was excised. The gel slice was rinsed with water and melted by heating in 100 µl water. About 2 µl of this solution was transferred to 200 µl of PCR buffer containing the modified F-TYRK/R-TYRK primers designed to include deoxy-UMP residues and a Not I restriction site at their respective 5′-ends (F-TYRKU: 5′-CUACUACUACUAGCGGCCGCAARRTNRSNGAYTTYGG-3′; R-TYRK: 5′-CAUCAUCAU-CAUGCGGCCGCCCRWANSHCCANACRTCNS-3′). This gave PCR products of the expected size (about 190 bp) suitable for treatment with Uracil DNA Glycosylase (UDG, Life Technologies) and cloning into pAMP1 (Gibco/LTI). Transformation was performed using MAX Efficiency DH5a competent cells (Gibco/LTI). Cells were incubated overnight at 37°C on LB plates containing 100µg/ml ampicillin, 50µg/ml X-gal and 1mM IPTG (Gibco/LTI). Ampicillin resistant clones were picked from agar plates and their insert sizes were determined by agarose gel analysis of PCR products generated with vector-specific PCR primers.

1.3 Perform pre-screening with known tk fragments, cDNA sequencing and database searches

The DNA from plasmid clones with inserts of about 125-190 bp was isolated, fingerprinted or screened against known tk genes and 'novel' clones were sequenced at the U.C. Berkeley, Biochemical Core Facility. The list of genes cloned and identified so far contained several sequences reported to have transforming activity, such as trk, axl/ufo or to be overexpressed in various types of cancer. Our present panel of kinase gene tags used to prepare cDNA micro-arrays contains 60 genes among them four novel sequences and HLA-A, which happened to be amplified by our PCR primers. In the reporting period, we noticed an elevated fraction of clones that were sequenced and revealed a previously sequenced insert, suggesting that our pre-screening procedure was not sufficiently stringent. For example, one batch sequencing procedure of 20 plasmid clones showed previously identified tk fragments in 18 out of 20 reactions, while the remaining 2 clones contained novel sequences without matches in the Genbank database.

1.4 Add novel clones to the panel of expressed tk gene fragments

As of June 2001, we cloned and partially characterized about 140 tk fragment containing plasmid clones derived from prostate cancer tissues and cell lines. These clones join more than 600 tk fragments containing clones that were previously isolated from thyroid and breast tumors. From these clones, DNA was isolated, bound to nylon filters and prescreened with probes prepared from known tk clones. Following this pre-screening step, we performed cDNA sequencing and database searches. In about 100 tk containing plasmids that were sequenced, we found two potentially novel prostate cancer related genes. The remaining clones contained sequences that were already part of the panel suggesting insufficient stringency during the pre-screening process. At this point, we decided to focus our efforts on the optimization of the PCR amplification (see 2.3, below). We initiated a search for full length cDNA clones for some of the novel tk sequences found expressed in prostate cancer. Employing a local company (Pangene Corp. of Fremont, CA) to screen their proprietary cDNA libraries for full length clones, we obtained a clone for one of our novel prostate cancer markers.

The clone named '404-53' carries an insert of 5.5kb in the 6.5 kb plasmid vector pEAK8. Sequence analysis of 1,316bp of the insert of clone 404-53 revealed high homology between a 1,248bp stretch of the insert with exons 72-74 of the human heparan sulfate proteoglycan 2.

Heparan sulfate proteoglycan 2 (HSPG2) (also called 'perlecan') is the major heparan sulfate proteoglycan of basement membranes. The gene is located on chromosome 1p36.1, a region that is frequently altered in human tumors. Perlecan possesses angiogenic and growth-promoting attributes primarily by acting as a coreceptor for basic fibroblast growth factor (bFGF) (Mundhenke et al., 2002). BFGF has diverse functions in cell growth and differentiation. In addition, multiple roles have been proposed for bFGF in cancer origin and progression. BFGF signals through transmembrane receptor tyrosine kinases. It is regulated by a balance of HSPGs that either stimulate or inhibit bFGF binding to its bFGF receptor. In breast cancer and prostate cancer, heparan sulfate proteoglycan might be involved in tumor progression (Mundhenke et al., 2002; Wu et al., 2001).

Task 2.1 Prepare DNA microarrays carrying about 100 different sequences

The fabrication of tyrosine kinase (tk) cDNA arrays on glass slides was done in house using our laboratory-built robotic system to print cDNA micro-arrays with about 124 target spots per array. The arrayer can print up to 91 slides in a single run. Routinely, we print approximately 50 slides per run, with duplicate arrays on each slide. The stainless steel printing pin has an open slit in the tip. Each loading takes up approximately 1 μl of DNA solution and allows continuous printing of more than 100 dots. A full 384-well plate of DNA samples can be arrayed onto 50 slides (in duplicate) in 8 hrs, including the time needed for repeated wash and drying steps in each printing cycle. We concentrated our PCR products and dissolved DNA in 50% dimethyl sulfoxide (DMSO) at a concentration of 250ng/μl. Spot diameters on poly-L-lysine coated slides ranged from 80-125 μm. The arrayer has a positioning precision of approximately 15μm, therefore, an array with 200μm center-to-center distance between spots is sufficiently spaced.

Standard microscope glass slides were cleaned thoroughly with concentrated sodium hydroxide and ethanol before being coated with poly-L-lysine. These steps provided a positively charged layer to bind DNA to the slides. The coated slides were stored in ambient air in closed slide boxes two weeks before they were used for printing. The locations of arrays were marked on the reverse side of the slides with diamond marker pen. The slides were placed upside down in a chamber filled with 1x SSC and re-hydrated for 5 to 15 minutes (depending on the size of the array). Spots are not allowed to swell too much and run into each other. Hydrated slides were dried on a hot plate set to 70 ^{0}C for 3 seconds. These slides were then UV cross-linked using a Stratalinker 1800 (Stratagene) set to 65 mJ. Following cross-linking, slides were incubated in blocking solution (0.21M succinic anhydride in 1-methyl-2-pyrrolidinone, 0.06M sodium borate for 20 minutes (pH 8.0)). The slides were then denatured in boiling water for 2 min, and submerged in 95% ethanol for 1 minute. After a centrifugation at 500 rpm for 5 min to remove traces of ethanol or water, the arrays were ready for hybridization or storage.

Quality control for the tk arrays was monitored throughout the tk array fabrication process. All reagents used for printing and coating of slides were prepared fresh and filtered. Before post-printing processing of the arrays, morphology of the spots and DNA distribution within the spots were examined by staining one or two slides from each batch with DAPI and visualized in a Zeiss fluorescence microscope. After post-printing processing, arrays were stained with POPO-3 dye (0.01 mM) and scanned at Cy3 channel to ensure the retention of DNA on the arrays for hybridization.

2.2 Optimize hybridization conditions to provide quantitative information

We isolated total RNA from prostate cancer cell lines or frozen tissues sections. We then prepared cDNAs from the RNA by random priming and reverse transcription using commercial kits (Qiagen, Roche, Ambion). Typically 1 μg of total RNA can produce sufficient quantities of probe for repeated micro-array analyses. We used about 100 ng of cDNA in PCR reactions to amplify tk-specific cDNA

fragments with our mixed-base F-/R-TYRK primers. Following 30 cycles of PCR, the amplification products were separated on a 4% agarose gel stained with ethidium bromide, and a slice that contained fragments of approximately 160-180 bp was excised. The gel slice was rinsed with water and melted by heating in 100 µl water. About 2 µl of this solution was transferred to 200 µl of PCR buffer containing the F-/R-TYRK primers. The tk fragments were then amplified a second time and the products concentrated via isopropanol precipitation. The tk fragments (about 400-600 ng per reaction (Hsieh et al. 2001)) were then labeled with either Cy3-dUTP (red fluorescence) or Cy5-dUTP (infrared fluorescence) by random priming (Gibco/LTI).

We optimized hybridization and wash conditions to obtain specificity and reduce background fluorescence to the level in which quantitative information could be obtained reproducibly. The performance of the system was tested by hybridization of fluorochrome-labeled tk gene-specific PCR fragments onto our tk-specific DNA micro-arrays. In preliminary experiments, we used a first generation of DNA micro-arrays comprised of 48 kinase genes previously identified to be expressed in thyroid tumors. This allowed us to optimize hybridization and wash conditions. We then prepared DNA micro-arrays carrying a more extensive panel of tk genes plus a few control genes. We found that hybridization to these arrays provided reproducible information relevant to tumor progression and differences between cell lines. In some of our experiments, we wanted to compare the expression levels of tk genes in a tissue sample with a standard mixture rather than a second tissue sample. Here, the small size of our array was an advantage. We created a reference standard to compare tk expression in a given tissue simply by mixing equimolar amounts of each tk spot on the array. This artificial mixture of DNAs is then labeled by random priming with Cy3-dUTP or Cy3-dUTP in an identical manner as the cDNAs derived from mRNA.

2.3 Optimize PCR parameters for quantitative amplification of target genes

We have worked out an effective method to generate probe for our microarray experiments. In our original method, we isolate total RNA from frozen tissues cell lines and prepared cDNAs from the RNA by random priming and reverse transcription, before we amplify tk-specific cDNA fragments with our mixed-base F-/R-TYRK primers.

In the course of our experiments, we have discovered that this probe synthesis method can generate quality microarray data. This method is particularly good at assaying the expression of tk genes that are poorly or moderately expressed. We subjected our results to independent validations and learned that our original method seems to recapitulate the results of other investigators (using Northern blots) for moderate and low expressing tk genes. However, we have also learned that highly expressed tk gene transcripts are less accurately represented in our experiments than we would prefer. The rank ordering of the high expressing genes is maintained (i.e. for tk genes that are highly expressed, a lower-expressed tk gene indeed appears to be expressed less than a higher-expressed tk genes). However, the apparent difference two highly expressed genes are less than one might expect. In essence, our original probe synthesis scheme is not completely representative of total tk expression.

We believe that the cause of this artifact is due to the depletion of degenerate primers in the early steps of PCR synthesis. Our degenerate primer mixes contain equimolar concentrations of each the hundreds of different primers. Of course, the population of tk RNAs within and tissues sample is likely to be anything but equimolar. It is probable that the most highly expressing genes are rapidly depleting the population of degenerate primers that would be perfect matches for that species. Once perfect primers are depleted, sub-optimal primers then bind with baspair mismatches leading to degrade PCR performance.

We have implemented revised methods to eliminate this problem. Our new PCR scheme utilizes new degenerate tk primers that have an unrelated 18-25 bp adapter sequence at its 5'-end and the tk – specific sequence on its 3'-end. The previous work of Eberwine (Eberwine, 1996) with a T7 adaptor sequence for in vitro transcription suggested that M13 or T7/T3 primer sequences on the 5'-ends works

well. The new degenerate primers are used for only a handful of PCR cycles with the cDNAs to initiate the amplification of the tk sequences. Then, the degenerate primers are removed with a spin column, and the adapter primers (representing the non-degenerate 5'-ends) are added to the products of the first PCR reaction. The adapter primers then complete the reaction. By allowing the degenerate primers access to our cDNA for only a very few cycles, we believe we can make our microarray results more representative. This will be even more important as we move to smaller samples sizes (in the following year). We have already tested different adaptor sequences, and are now in the process to compare the complexity of PCR products generated with either to old or the new amplification strategy.

We modified our primer design to more specifically amplify tyrosine kinases sequences when preparing probes for our expression micro-array. The original primer design was based on two highly conserved amino acid sextets approximately 40-55 amino acids apart (Figure 1). While these primers amplified the tyrosine kinase target mRNAs, they amplified other sequences as well. Our strategy was to design a new primer set focused on the conserved mRNA sequence of the tyrosine kinase domain rather than of the protein sequence. In this way, we could eliminate primers from the degenerate pool that had no chance of amplifying tyrosine kinase sequences

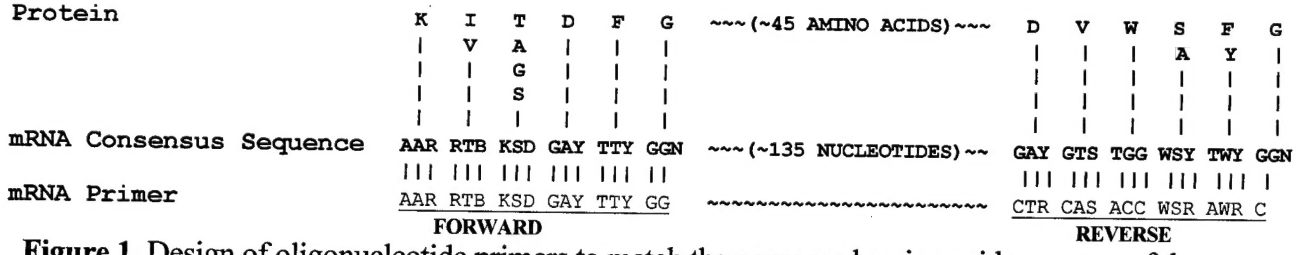

Figure 1. Design of oligonucleotide primers to match the conserved amino acid sequence of tk genes.

All protein tyrosine kinases mRNAs were obtained through the National Center for Biotechnology and aligned by DIALIGN version 2 (Morgenstern, 1999). The aligned sequence results were input into GeneFisher version 1.22 to generate a consensus sequence. The nucleotides corresponding to the two highly conserved amino acid sextets were identified, which led to the generation of the new forward and reverse primers, AAR RTB KSD GAY TTY GG and CRW ARS WCC ASA CRT C, respectively. Although the new primer set is similar to the previous set, the complexity of the new primers was decreased. The degeneracy of the new forward primer was decreased 1.8 fold (from 1024 to 576) while the reverse primer was decreased 3 fold (from 6144 to 2048).

In anticipation of the addition of other tyrosine kinase genes on our next generation of expression microarray, we obtained mRNA sequences of an additional 50 tyrosine kinases and used computer modeling to determine if the primers would be able to amplify the tyrosine kinase domain of the additional tyrosine kinases. All 50 tyrosine kinase genes were alignmented and a consensus sequence was obtained. Of the 50 additional tyrosine kinase genes, 45 had good alignment according to DIALIGN version 2 and should amplify the tyrosine kinase domains of the additional genes.

2.4 Develop algorithms for array readout and comparisions between measurements

A Axon GenePix 4000 (Axon Inc.) array scanner used to acquire all images has a preview resolution of 40 μm and a scanning resolution of 10 μm. The analysis software provided numerical data that was imported directly into spreadsheets (Microsoft Excel) for further analysis. For display purposes, the images were saved in standard format (tiff 6.0) and imported into common graphics programs such as Adobe Photoshop. Given the relatively small numbers of arrays that have been hybridized so far, it was sufficient to normalize the data and compare numeric values in spreadsheets.

Task 3. **Validate assays for multigene expression profiling in small amounts of tissue**

3.1 Develop software for databasing, automated analysis of expression profile datasets and their annotation

The GenePix software provides the results as numeric data that can be inported into spreadsheets or databases. We installed Microsoft Access software which will be sufficient to handle most or all of our databasing needs. We also installed BRB ArrayTools Version 1.03 recently released by the Biometric Research Branch of the Division of Cancer Treatment and Diagnosis at NCI. This package developed by Richard Simon and Amy Peng allows for a more comprehensive analysis of DNA microarrays and their annotation. The software is presently being tested using the datasets obtained with our second generation tk cDNA arrays.

One of the key goals in our project is to minimize the amount of tissue required to create probe for our arrays. With the original tyrosine kinase degenerate primers, we were able to able to visualize the RT-PCR products from the total RNA of 200 cell equivalents. We have recently used our new lower-complexity primers to visualize RT-PCR products total RNA. We are readily able to detect RT-PCR products from 2000 cell equivalents and barely detect products from 200 cell equivalents. The reduction in signal is probably due to a sharp reduction in the levels of non-specific products amplified by the new primers. Although we have not reached the goal of single cell tyrosine kinase profiling, we still have many options available to help achieve it. A recent technical advance is one-step RT-PCR kits that combine reverse transcription and DNA amplification. The sharply reduced need for human handling should allow for very rapid optimization and more sensitive RT-PCR.

KEY RESEARCH ACCOMPLISHMENTS:

- Finished the isolation of RNA and synthesis of cDNA from 15 prostate cell lines and 16 frozen tissue specimens
- Completed the PCR-amplification of tk-specific DNA fragments and cloned the products
- Screened more than 300 prostate cancer cell line-derived clones and sequenced an additional 100 clones, database searches identified two clones containing potentially novel genes with relevance to prostate cancer progression
- Expanded the panel of tyrosine kinase genes used for expression profiling and printed second generation cDNA micro-arrays
- Reconfirmed tk gene expression changes as prostate epithelial cells become tumorigenic and grow in nude mice using second generation tk micro-arrays
- Redesigned our oligonucleotide primers to increase specificity and processivity
- Obtained a full length cDNA clone for one of our abnormally expressed genes

REPORTABLE OUTCOMES:

- peer reviewed publications

1. Weier H-UG, Greulich-Bode KM, Ito Y, Lersch RA, Fung J (2002) FISH in cancer diagnosis and prognostication: from cause to course of disease. Expert. Rev. Mol. Diagn. 2(2):109-119
2. Weier, H.-U.G. (2002) Quantitative DNA Fiber Mapping. In: FISH Technology. B. Rautenstrauss and T. Liehr (Eds.), Springer Verlag, Heidelberg, pp.226-253
3. Lersch RA, Fung J, Hsieh H-B, Smida J, Weier H-UG (2001) Monitoring signal transduction in cancer: from chips to FISH. J Histochem Cytochem 49:925-926
4. Hsieh H-B, Lersch RA, Callahan DE, Hayward S, Wong M, Clark OH, Weier H-UG (2001) Monitoring signal transduction in cancer: cDNA microarray for semi-quantitative analysis. J Histochem Cytochem 49:1057-1058
5. Weier H-UG, Zitzelsberger HF, Hsieh H-B, Sun MV, Wong M, Lersch RA, Yaswen P, Smida J, Kuschnick C, Clark OH (2001) Monitoring signal transduction in cancer: tyrosine kinase gene expression profiling. J Histochem Cytochem 49:673-674

- abstracts submitted

2002 Annual Meeting of the Histochemical Society, Seattle, WA, July 18-21, 2002:

1. Lersch RA, Chu LW, Ito Y, Weier HUG. Toward Tyrosine Kinase Expression Profiling at the Single Cell Level.
2. Weier HUG, Ito Y, Fung J, Lehmann L, Lersch RA, Chu LW, Zitzelsberger HF. Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history.
3. Ito Y, Fung J, Hsu J, Katzir N, Lersch RA, Weier HUG. Phenotype analysis of tumor cells with eight color FISH.

- funding obtained

Supplement to 'Spectral Karyotyping for Phenotype Analysis of Cancer Cells', National Institute of Health, R33 CA80792, HUG Weier (P.I.), 10/01/01-9/30/03

CONCLUSIONS:

This 3-year IDEA project is well on track and, has met its initial milestones. The soft- and hardware components necessary for these studies were put in place in the first year. The results obtained with RNA isolated from cell lines and prostate tissues have proven the hypothesis that changes in tk gene expression can be monitored by a combination of PCR using tk gene family-specific primers and DNA micro-arrays. While the hybridization to the DNA micro-array appears to possess the required specificity, second year research addressed the issues of hybridization background reduction and definition of a suitable reference DNA probe. Comparison of the cDNA micro-array data with those obtained by Southern blot analyses suggested a non-homogeneous amplification of tk fragments. This is now addressed by an altered PCR protocol involving newly designed primers. Concordant with the timeline presented in the original proposal, research and development in the third year will focus on application of the technology to a small number of cells.

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Wu X, Kan M, Wang F, Jin C, Yu C, McKeehan WL (2001) A rare premalignant tumor epithelial cell syndecan-1 forms a fibroblast growth factor-binding complex with progression-promoting ectopic fibroblast growth factor receptor1. Cancer Research 61: 5295-5302.

APPENDICES:

1. Lersch RA, Chu LW, Ito Y, Weier HUG. Toward Tyrosine Kinase Expression Profiling at the Single Cell Level.
2. Weier HUG, Ito Y, Fung J, Lehmann L, Lersch RA, Chu LW, Zitzelsberger HF. Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history.
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4. Weier H-UG, Zitzelsberger HF, Hsieh H-B, Sun MV, Wong M, Lersch RA, Yaswen P, Smida J, Kuschnick C, Clark OH (2001) Monitoring signal transduction in cancer: tyrosine kinase gene expression profiling. J Histochem Cytochem 49:673-674
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TOWARD TYROSINE KINASE EXPRESSION PROFILING AT THE SINGLE CELL LEVEL

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cDNA microarray technology can provide an extremely detailed RNA expression profile of tissues. To be maximally useful in research or medicine, expression profiling should be extended to individual cells. Cell-by-cell RNA profiles would allow researchers to tackle difficult issues of tissue heterogeneity. Based on our experience cloning tyrosine kinase genes, we have developed a PCR-based method that allows us to determine the expression profile of tyrosine kinase genes in a tissue. This method relies on degenerate primers designed to amplify a region between two relatively well-conserved domains within a tyrosine kinase transcript. The primer binding sites have been selected to be approximately the same distance apart so the PCR products can be visualized and purified to greatly improve probe specificity. The amplified sequences between priming sites are sufficiently divergent to determine an expression profile by DNA microarray hybridization. Since our method relies on well-understood PCR techniques, it can be extended to single cell analysis. We are currently refining this method to make a representative tyrosine kinase profile of a single cell. We view this single cell profiling as an essential complement to our efforts in phenotypic analysis with multi-color FISH (see abstract by Ito et al.). Furthermore, since our method relies upon making degenerate primers to sequences that define a gene family, we believe that this approach is generally applicable to profile other gene families such as serine/threonine kinases, transcription factors, and other critical regulators of cellular control that have conserved domains.

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Chromosome rearrangements in a cell line derived from a case of childhood papillary thyroid cancer (chPTC) with radiation history

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Papillary thyroid cancer occurs in sporadic or familial forms as well as after exposure to ionizing radiation. Although these tumors appear to be caused by different genetic or external factors, many of them share a surprising phenotypical similarity. Few genes are known to be expressed abnormally in chPTC with radiation history. The most common rearrangement leads to abnormal expression of the catalytic domain of the ret proto-oncogene, a tyrosine kinase (tk) gene that maps to chromosome 10q11.2. Searching for other abnormally expressed genes involved in the chPTC phenotype, we applied molecular cytogenetic methods to the investigation of chromosome rearrangements in the chPTC cell line S48. This cell line forms tumors in nude mice, yet tested negative for expression of normal or rearranged copies of ret. G-banding analysis suggested complex rearrangements and the presence of several marker chromosomes. Comparative genomic hybridization indicated gains and losses of parts of several chromosomes, most notably chromosomes 1, 2, 6, 9, 13, and 22. These structural alterations were confirmed by Spectral Karyotyping which indicated only a single reciprocal translocation in the presence of several more complex rearrangements. A combination of positional cloning and candidate gene approaches will lead to a better characterization of chromosomal breakpoints, and the discovery of two rearranged forms of another tk gene, the tropomyosin receptor kinase NTRK1.

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Phenotype analysis of tumor cells with eight color FISH

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High throughput gene expression profiling using cDNA microarrays generates a wealth of information and often demonstrates tumor-specific changes. These measurements, however, provide average values for tumor cell populations that may be rather heterogeneous. Our technical developments address the issue of heterogeneity in tumor research by developing an analytical system capable of performing semi-quantitative multi-gene expression profiling of single cells. Targeting cell-by-cell measurements of expression levels of multiple tumor markers, our approach uses RNA/cDNA fluorescent in situ hybridization (FISH) combined with Spectral Imaging and digital image analysis. While the system is capable of deconvoluting images of objects stained with up to nine fluorochromes, we performed initial tests of system resolution and reproducibility with commercially available beads fluorescing in seven different wavelength intervals. The system measured up to our expectation of being able to quantitate the seven different fluorescent reporter molecules with relative standard deviations ranging from 1% to 6.1%. Using eight different fluorochromes, we then analyzed the expression levels of 6 different tyrosine kinase gene and one genomic target in breast and thyroid cancer cells counterstained with DAPI. In artificial mixtures, the system was able to recognize the tumor cells based on the level of expression of one or two genes, and could identify cells present in only a few percent.

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BRIEF REPORT

Monitoring Signal Transduction in Cancer: Tyrosine Kinase Gene Expression Profiling

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SUMMARY Abnormal expression of tyrosine kinase (TK) genes is common in tumors, in which it is believed to alter cell growth and response to external stimuli such as growth factors and hormones. Although the etiology and pathogenesis of carcinomas of the thyroid or breast remain unclear, there is evidence that the expression of TK genes, such as receptor tyrosine kinases, or mitogen-activated protein kinases, is dysregulated in these tumors, and that overexpression of particular TK genes due to gene amplification, changes in gene regulation, or structural alterations leads to oncogenic transformation of epithelial cells. We developed a rapid scheme to measure semiquantitatively the expression levels of 50–100 TK genes. Our assay is based on RT-PCR with mixed based primers that anneal to conserved regions in the catalytic domain of TK genes to generate gene-specific fragments. PCR products are then labeled by random priming and hybridized to DNA microarrays carrying known TK gene targets. Inclusion of differently labeled fragments from reference or normal cells allows identification of TK genes that show altered expression levels during malignant transformation or tumor progression. Examples demonstrate how this innovative assay might help to define new markers for tumor progression and potential targets for disease intervention. **(J Histochem Cytochem 49:673-674, 2001)**

KEY WORDS
tyrosine kinase
tumors
RT-PCR
genes

BECAUSE the malignant transformation of epithelial cells and progression of carcinomas are accompanied by changes in the expression of receptor and cytosolic tyrosine kinase (TK) genes, we set out to develop an innovative DNA microarray-based assay to simultaneously determine the relative expression level of 50–100 TK genes using just a few cells.

A number of studies have shown that tumor development is accompanied by at least two changes: (a) a change in the way cells interact with their environment via membrane-bound receptors, and (b) a change in how signals originating from these receptors are transduced from the cell membrane to the cytoplasm and the nucleus. Among the hundreds of genes involved in receptor-mediated signal transduction, only a few are aberrantly expressed in tumors. A major motif in signal transduction is the selective phosphorylation and dephosphorylation of tyrosine residues in protein factors involved in signal processing. Proteins that phosphorylate tyrosine residues are products of genes belonging to the family of TK genes. The number of known TK genes has grown steadily in recent years, and the temporal and tissue-specific expression of ~100 different TK genes in normal cells is carefully orchestrated. The level of expression of certain TK genes is increased in many human tumors (Luttrell et al. 1994; Liu et al. 1995). Simple overexpression of TK genes due to gene amplification or to changes in the regulation of gene expression may lead to oncogenic transformation. This has been clearly documented for the erbB2 protein, the product of the Her-2/neu proto-oncogene and other members of the erb B

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family (Tse et al. 1997). In addition, many tumors have acquired structurally altered TK proteins or abnormal expression patterns through de novo mutational events. When chromosomes became rearranged, the catalytic domain of a TK gene was found fused to the amino terminal of another protein, thus creating a new transforming activity as well as a new expression pattern. Well-known examples of this mechanism of oncogene activation are the bcr/abl-fusion protein in chronic myeloid leukemia with a translocation, t(9;22), and the activation of the receptor TKs ret and trk in papillary thyroid cancer (Sozzi et al. 1992; Jhiang and Mazzaferri 1994).

Various protein factors can be mis-expressed and, in combination with other events, might constitute one of several factors leading to the onset and/or progression of cancer. Factors including cell cycle-specific enzymes, hormone receptors, and peptide growth factors have been reported as having prognostic significance in some cases of prostate cancer. Overexpression of particular receptor TK genes such as the insulin-like growth factor receptors (IGF-IRs), the epidermal growth factor receptor (EGFR or erb B) family of receptors, focal adhesion kinase (FAK), or the proto-oncogenes ret and Nyk/mer have been shown to correlate with progression to a more malignant phenotype in a variety of tumors (Resnik et al. 1998), among them carcinoma of the prostate (Ling and Kung 1995; Dawson et al. 1998). Detailed knowledge about TK gene expression and its relation to tumor progression might increase our understanding of how tumors grow and help us design assays to more accurately stage tumors.

Our project targets the development of a novel assay format that enables us to determine the level of expression of many different genes. A rapid assay uses DNA microarrays carrying small amounts of individual TK gene-specific targets to simultaneously determine the expression level of up to 100 TK genes using a small number of cells. We cloned and characterized TK genes expressed in thyroid cancers and in seven different breast cancer cell lines. Using mixed-base oligonucleotides specific for conserved domains in the catalytic domains of TK genes, our PCR assays amplified ~159–171-bp fragments of expressed TK genes. We size-selected and cloned the PCR products into plasmids. As of August 2000, we had identified more than 50 TK genes expressed in thyroid cancers plus an additional 172 TK fragment clones derived from breast cancer cell lines and ~250 TK fragment clones from radiation-induced thyroid cancers in the prescreening and sequencing steps. Previously, we finished the construction of a robotic system to print DNA microarrays with about 100 sequences on glass slides (unpublished data). The performance of the system was tested by hybridization of fluorochrome-labeled TK gene-specific PCR fragments onto the TK-specific DNA microarrays. All experiments involving human cells or cell lines were approved by the U.C. Berkeley Human Subject Use Institutional Review Board. These experiments enabled us to optimize hybridization and washing conditions and to generate data regarding the relative level of gene expression. For example, the 184A1 and 184A1TH cell lines are closely related non-tumorigenic and tumorigenic human mammary epithelial cell lines derived from the same normal breast tissue specimen and transformed in vitro. After hybridizing Cy5-labeled TK fragments prepared from the cell line 184A1 and Cy3-labeled TK fragments prepared from cell line 184A1TH to a DNA microarray carrying 58 TK gene fragments and displaying Cy3 fluorescence signals in green and Cy5 signals in red, differences between the cell lines became readily apparent. Genes expressed at a higher level in 184A1TH cells led to increased green signals on the array, while those genes whose expression level is lower in 184A1TH cells compared to 184A1 cells generated spots that exhibited stronger red fluorescence. Similarly, differences between thyroid tumor cell lines could be demonstrated in simple dual-color hybridization experiments.

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BRIEF REPORT

Monitoring Signal Transduction in Cancer: cDNA Microarray for Semiquantitative Analysis

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SUMMARY This study targeted the development of a novel microarray tool to allow rapid determination of the expression levels of 58 different tyrosine kinase (tk) genes in small tumor samples. The goals were to define a reference probe for multi-sample comparison and to investigate the variability and reproducibility of the image acquisition and RT-PCR procedures. The small number of tk genes on our arrays enabled us to define a reference probe by artificially mixing all genes on the arrays. Such a probe provided contrast reference for comparative hybridization of control and sample DNA and enabled cross-comparison of more than two samples against one another. Comparison of signals generated from multiple scanning eliminated the concern of photo bleaching and scanner intrinsic noise. Tests performed with breast, thyroid, and prostate cancer samples yielded distinctive patterns and suggest the feasibility of our approach. Repeated experiments indicated reproducibility of such arrays. Up- or downregulated genes identified by this rapid screening are now being investigated with techniques such as in situ hybridization.

(J Histochem Cytochem 49:1057–1058, 2001)

KEY WORDS
cDNA microarray
expression profiling
tyrosine kinase
reference probe

Our DNA microarray experiments followed the Brown laboratory's protocols (http://cmgm.stanford.edu/pbrown/mguide/index.html) with modifications on microarrayer construction and the following: spotted DNAs are homogeneous (270–290 bp); RNAs were amplified by RT-PCR with mixed-base tk primers, size-selected by electrophoresis (180–210 bp), and labeled by random prime reaction. Typical cDNA microarray experiments utilize two-color hybridization in which Cy5-labeled sample is hybridized against Cy3-labeled control to the same arrays (Schena 1996). This provides a convenient means to look at two treatment effects, such as two time points (Iyer et al. 1999) or tumor vs normal tissues (DeRisi et al. 1996). However, when multiple samples are to be compared against one another, pair-wise comparisons are inefficient. To address such needs and reduce the complexity, a "standard" or "reference" probe is preferred. Because the hybridization is competitive with limited labeled DNA (Cheung et al. 1999), a pooled mixture that represents all genes at low (but detectable) levels will be an ideal reference. For large-format cDNA microarray, pooled mixtures of several normal tissues or cell lines were used (Celis et al. 2000). Such pools of RNA can vary from preparation to preparation, and highly expressed genes can skew or saturate the arrays. With a small number (58) of genes on our tk arrays, we were able to artificially mix individual genes proportionally and to amplify them in large quantity for a particular project. Pair-wise hybridizations with this reference probe have yielded similar results that proved this approach feasible.

To test the extent of the scanner's intrinsic noise, slides were scanned 12 times repeatedly at full laser power. Four separate pixels belonging to four intensity groups were selected and their mean, median, SD, and CV were determined (Table 1). Results indicated an average of 5.0% and 6.0% CV for Cy5 and Cy3 channels, respectively. Noises were negligible and did not correlate with PMT voltages; the deviations of measured intensities were within experimental errors ($<<10\%$). Repeated scanning up to 24 times did not decrease the fluorescence intensity, eliminating the concern of photo bleaching.

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Table 1 Repeated scanning of the same array 12 times at full laser power. Results for four different pixels in Cy5 and Cy3 channels indicated negligible photo bleaching and <<10% coefficients of variation

Pixel #	1		2		3		4	
Coordinate	X = 12390 Y = 9420		X = 12600 Y = 9420		X = 12790 Y = 9420		X = 12980 Y = 9630	
	Cy5	Cy3	Cy5	Cy3	Cy5	Cy3	Cy5	Cy3
50% PMT								
Mean	1027.4	3545.9	416.7	702.3	1966.3	2112.2	2215.5	1257.6
Median	1024.5	3534.5	409.5	708.5	1951.0	2103.0	2219.0	1261.0
SD	46.1	151.5	27.8	40.6	105.2	108.8	89.8	66.1
CV (%)	4.5	4.3	6.7	5.8	5.3	5.2	4.1	5.3
70% PMT								
Mean	13035.1	44865.2	5102.4	8514.1	25737.5	26482.1	29591.7	15903.5
Median	12969.5	45105.5	5135.5	8381.0	28009.5	28677.0	29519.5	15833.5
SD	537.4	2384.0	309.9	671.6	1550.0	2254.0	1514.2	554.9
CV (%)	4.1	5.4	6.1	7.9	6.1	8.5	5.1	3.5
75% PMT-Cy5, 70% PMT-Cy3								
Mean	19371.4	40990.5	7277.1	7487.8	36491.5	24824.8	41453.4	15200.2
Median	19359.5	41011.5	7366.0	7491.0	36285.5	25081.0	41727.0	15295.0
SD	511.8	2779.5	485.2	652.1	1310.1	1090.6	2236.6	954.6
CV (%)	2.6	8.8	8.7	8.7	3.6	4.4	5.4	6.3

Two properties regarding the RT-PCR amplification were examined: Do the amplified probes represent the true abundance of each transcript in cells? Does the probe saturate the arrays? First, we mixed five tk genes in three ratios (1, 20, and 400) and used them as PCR templates. Hybridizations of such PCR product to a small five-tk gene array showed a "compressed" ratio of three- to fourfold despite a 400-fold input template ratio. Nevertheless, the relative order of abundance of the five tk templates was conserved. Saturation has likely occurred in PCR, which may have also depleted some tk primers due to their high degeneracy. Second, we compared single-probe hybridizations at various concentrations. Four to six hundred ng of PCR amplified products typically were used for random prime labeling. The labeled probe was purified and different dilutions were applied to the arrays. It was determined that one tenth of the labeled probe per channel provided optimal signals for analysis without saturating the arrays.

Repeated hybridizations have identified distinctive patterns among thyroid, breast, and prostate cancer cell lines (Figure 1). Although the panel of 58 tk genes may not represent all the tk expressed in any single cancer tissue, we have reason to believe they represent over 50% of human tk genes expressed in a particular tissue type (Robinson et al. 1996). These distinctive expression patterns, as unveiled by microarray, become molecular fingerprints of tk gene expression for each tumor.

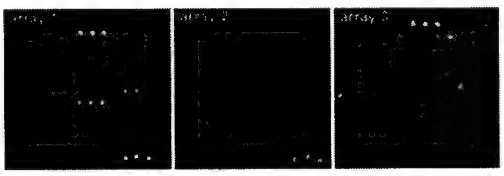

Figure 1 Multiple hybridizations confirmed distinctive patterns for three tumor cell lines. **(Array 1)** Prostate cancer cell lines Cy5:BPH-1-CAFTD-04/Cy3:BPH-1-CAFTD-02. **(Array 2)** Thyroid cancer cell lines Cy5:FTC133/Cy3:FTC236. **(Array 3)** Breast cancer cell line Cy5:MCF7/Cy3:58 tk mix.

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BRIEF REPORT

Monitoring Signal Transduction in Cancer: From Chips to FISH

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SUMMARY The microarray format of RNA transcript analysis should provide new clues to carcinogenic processes. Because of the complex and heterogeneous nature of most tumor samples, histochemical techniques, particularly RNA fluorescent in situ hybridization (FISH), are required to test the predictions from microarray expression experiments. Here we describe our approach to verify new microarray data by examining RNA expression levels of five to seven different transcripts in a very few cells via FISH. (J Histochem Cytochem 49:925–926, 2001)

KEY WORDS
spectral imaging
FISH
microarray
RNA
cancer

OUR LABORATORY is adapting microarray technology to identify new genes involved in the formation of tumors in the thyroid, breast, and prostate. Previously, the transcription levels of only a few genes could be assayed per experiment. Microarrays circumvent this limitation. However, a second problem limits progress in cancer research. Most tumors are a mix of cell types, due in part to the normal complexity of the tissue and in part to the complexity of tumors as they evolve from benign to metastatic. If researchers collect microarray data without confronting the problem of tumor heterogeneity, important correlations will be missed.

Preliminary studies performed in our lab and elsewhere indicated that solid tumors are heterogeneous with respect to oncogene expression. Figure 1 shows the expression of brk (O'Bryan et al. 1991) in a childhood thyroid cancer that arose after the Chernobyl nuclear accident (Zitzelsberger et al. 1999). In this case, two cells strongly express brk (arrows point to their nuclei) and a nearby cell does not express brk. These studies were performed with touchprep papillary thyroid tumor specimens using filter-based microscope systems and only two different cDNA probes. If more than two probes are used per experiment, more slides, time, and reagents must also be used. All experiments involving human cells or cell lines were approved by the U.C. Berkeley Human Subject IRB.

To address this problem, we are developing a system to simultaneously measure cell-by-cell RNA levels of several different markers. The proposed scheme to discriminate benign and malignant neoplasms and to identify new prognostic markers will take advantage of new methods and our experience with oncogene activation in thyroid tumors. Our short-term goal is to develop a system which determines the relative level of expression of five tyrosine kinase genes using FISH-based methods and Spectral Imaging (SIm). Existing SIm instrumentation can record fluorescence spectra from 400 nm to 1100 nm with about 10-nm resolution, whereas the resolution of a light microscope is about 1 μm.

SIm combines the techniques of fluorescence microscopy, charge-coupled device (CCD) camera, and Fourier spectroscopy. The light emitted from each point of the sample is collected with the microscope objective and sent to a collimating lens. This light travels through an optical head (interferometer), is focused on a CCD camera, and the resulting data are processed with a computer. The interferometer divides each incoming beam (light from the microscope) into two coherent beams and creates a variable optical path difference (OPD) between them. The beams are then combined to interfere with each other. The resulting interference intensity is measured by the CCD detector as a function of the OPD. The intensity vs

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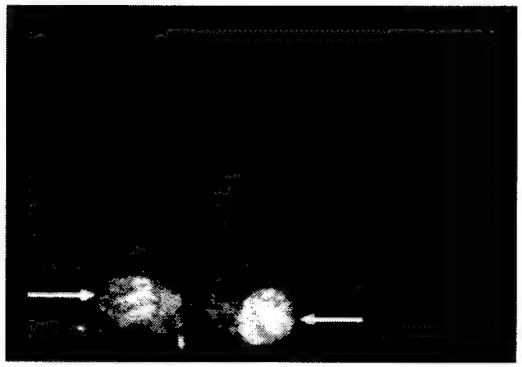

Figure 1 Frozen tissue touchprep of childhood thyroid tumor (S246) was hybridized with a cDNA probe for brk. The probe was labeled with digoxigenin and detected with rhodamine-conjugated antibodies. About 5% of the cells expressed brk at high levels, the other cells at low levels. Arrows indicate two high brk-expressing cells.

OPD is an "interferogram." The spectrum, i.e., intensity, as a function of wavelength can be recovered from the interferogram using Fourier transforms. The first published application of SIm (spectral karyotyping or SKY) (Schroeck et al. 1996) was developed to screen metaphase spreads for translocations with 24 chromosome-specific whole chromosome painting probes labeled with Spectrum Green, Spectrum Orange, Texas Red, Cy5, or Cy5.5, or combinations thereof. The multiple bandpass filter set (Chroma Technology; Brattleboro, VT) used for fluorochrome excitation was designed to provide broad emission bands (giving a fractional spectral reading from ~450 nm to ~850 nm).

Although we can use much of the existing technology, the ratio-labeling color scheme for SKY will not work for RNA detection because different labeled cDNA probes might co-localize. Therefore, we will label each cDNA probe with a unique reporter. The fluorescence spectra of the different reporter molecules can partially overlap, because the signals can be resolved by a computer algorithm termed "Spectral UnMixing (SUN)" (Applied Spectral Imaging; Carlsbad, CA). SUN enables us to deconvolute overlapping spectra and recover single component images from the spectral image.

The existing commercial SKY metaphase chromosome analysis software will be modified to increase its automated signal processing, RNA identification in interphase cells, integration of cDNA probe signals, and databasing of results. We will only refine the analysis software; the acquisition methods of capturing a spectral image and a separate high-contrast monochrome DAPI image will remain unchanged. A prototype of this system for analysis of protein markers has already been developed (Tsurui et al. 2000) and will aid our efforts to analyze RNA transcript levels.

Initially we will hybridize cDNA probes to brk, ret, c-met, trk, axl/ufo (obtained from Research Genetics; Huntsville, AL) using five cyanine dyes (Amersham; Arlington Heights, IL). On the basis of previous microarray results, we will also add members of the Eph tyrosine kinase family. cDNA probes will be prepared by incorporating fluorochrome-labeled deoxynucleoside triphosphates by random priming or PCR amplification (Weier et al. 1994,1995). We will also build a reference spectra database for these probes. Standard fixation and hybridization protocols should detect the target RNAs. It is unclear if we will need to prepare custom blocking agents to DNA repeats in the 3′-untranslated region of some of our targets. Using artificial mixtures of existing cell lines, we will develop software modules needed to measure intracellular levels of five RNA species and to determine important process parameters: sensitivity, accuracy, and reproducibility.

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Quantitative DNA Fiber Mapping

Heinz-Ulrich G. Weier

Introduction

High resolution physical maps have become indispensable for the positional cloning of disease genes and large scale sequencing projects. Common maps are based on ordered sets of clones from sources such as cosmid, P1/PAC/ BAC, or yeast artificial chromosome (YAC) libraries. The assembly of such maps is facilitated by application of fluorescence in situ hybridization (FISH). Hybridization of non-isotopically labeled probes onto preparations of DNA molecules ('DNA fibers') that were bound with one or both ends to a solid substrate and stretched homogeneously, forms the base of our 'Quantitative DNA Fiber Mapping (QDFM)' technique. Because the DNA fibers are easily accessible to probes and detection reagents, hybridization efficiencies are typically high and allow DNA targets as small as 500-1000 bp to be detected routinely. Quantitative DNA fiber mapping experiments require only standard laboratory equipment and access to a fluorescence microscope. By hybridizing one clone onto another, the extent and orientation of overlaps can be quantitated with near kilobase resolution. To measure the physical distance between non-overlapping DNA fragments, probes are hybridized to DNA fibers representing a larger genomic interval (Fig. 1). This also allows the mapping of expressed sequences (cDNAs) along DNA fibers representing genomic DNA.

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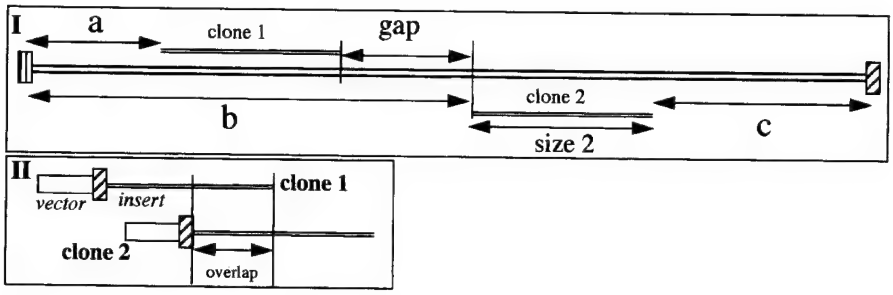


Fig. 1. Physical mapping strategies. I Mapping a small DNA molecule on to a larger molecule; II mapping of clone overlap by pairwise hybridization

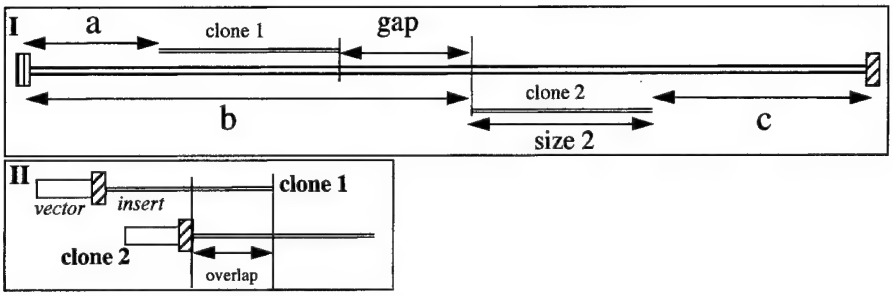
Outline

- Isolate DNA
- Pretreat microscope slides or coverslips
- Prepare DNA fibers on glass
- Select probe set
- Denature and hybridize probe set
- Remove unbound probe
- Detect bound probes with antibodies
- Acquire images
- Analyze images
- Save results in spreadsheets for further analysis

Principles and applications

High resolution physical maps have proven indispensable for large-scale, cost-effective gene discovery. Knowledge about the extent of overlap between any two clones and the precise localization of cloned DNA fragments within much larger genomic fragments is needed to assemble such maps. As demonstrated in this chapter, FISH can provide this critical information.

Isolation of DNA from cell nuclei and preparation of some sort of chromatin 'fibers' improves the accessibility of the DNA targets for probes as



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well as detection reagents, and thus increases the hybridization efficiency. Furthermore, if the DNA molecules can be stretched in some way, they may provide linear templates for visual mapping. FISH applied in the past to various types of crude DNA preparations allowed visualization of probe overlap and it provided some information about the existence and size of gaps between clones. However, none of those techniques provided sufficiently accurate information about the extent of clone overlap or the separation between elements in the map because the chromatin onto which clones were mapped was condensed to varying degrees from site to site.

We demonstrated previously that cloned DNA fragments can readily be mapped by FISH onto DNA molecules prepared by the hydrodynamic action of a receding meniscus and, referring to its quantitative nature, we termed the technique 'Quantitative DNA Fiber Mapping (QDFM)' (Weier et al. 1995). In QDFM, a solution of DNA molecules is placed on a glass or mica surface prepared so that some DNA molecules attach at one or both ends. The DNA solution is then spread over a larger area by placing a coverslip on top, and additional DNA molecules are allowed to bind to the surface. During drying, the molecules are straightened and uniformly stretched by the hydrodynamic action of the receding meniscus. Molecules prepared in this manner are stretched with remarkable homogeneity. A properly stretched molecule should extend about ~2.3 kb/μm, i.e., approximately 30% over the length predicted for a double stranded DNA molecule of the same size (Weier et al. 1995). QDFM can be applied to DNA molecules ranging in size from a few kb to more than 1 Mbp, which allows mapping of small probes with near kilobase resolution onto entire yeast chromosomes and large (mega)YAC clones (Wang et al. 1996, Duell et al. 1997).

Applications of QDFM extend beyond map assembly and can provide valuable information for quality control, clone validation, definition of a minimal tiling path as well as for the sequence assembly process. Furthermore, due to its high hybridization efficiency obtained with DNA fibers, QDFM is also the method of choice for high resolution optical mapping of expressed sequences in genomic intervals defined by the DNA fibers.

Materials

Equipment

- Refrigerated Centrifuge (MP4R, IEC)
- Dry bath (Model 2001, Labline Instruments)
- Fluorescence microscope (Axioskop, Zeiss) equipped with 40× and 63× oil immersion lenses
- Incubator oven (set to 37°C, Precision Scientific)
- Pulsed Field Gel Electrophoresis (PFGE) system (Biorad)
- Shaking Incubators (New Brunswick): 30°C for yeast cell culture, 37°C for culture of *E. coli*
- Thermal cycler for in vitro DNA amplification
- Water bath (Model 188, Precision Scientific)
- Digital Imaging system (optional)

Reagents

- 3-Aminopropyltriethoxy silane (APS, Sigma Chemicals)
- β-Mercaptoethanol (Sigma Chemicals)
- β-Agarase (New England Biolabs (NEB))
- Agarose (Life Technologies)
- Antibodies against digoxigenin, rhodamine-conjugated, made in sheep (Roche Molecular Biochemicals), stock solution is 1 mg/ml in PNM, dilute 1:50 with PNM prior to use. Store at 4°C.
- Antibodies against FITC, made in mouse (DAKO), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4°C.
- Anti-mouse antibodies, FITC conjugated, made in horse (Vector Labs), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4°C.
- Anti-avidin antibodies, biotinylated, made in goat (Vector Labs), stock solution is 1 mg/ml in PNM, dilute 1:50 prior to use. Store at 4°C.
- Avidin conjugated to AMCA (Vector Labs), stock solution is 2 mg/ml in PNM, dilute 1:500 prior to use. Store at 4°C.
- Blocking reagent: cat. #1096-176 (Roche Molecular Biochemicals)
- Chloroform/isoamyl alcohol: 24:1 vol:vol. (Life Technologies)

- 4, 6-diamino-2-phenylindole (DAPI) (Calbiochem), 0.05 μg/ml in antifade solution. Store at -20°C.
- dATP, dCTP, dGTP, dTTP: 100 mM each (Roche or Pharmacia). Store at -20°C.
- Digoxigenin-11-dUTP: 1 mM (Roche Molecular Biochemicals). Store at -20°C.
- 10× dNTP mix: dATP, dCTP, dGTP, and dTTP, 10 mM each.
- EDTA (ethylenediamine tetraacetic acid): 0.5 M (pH 8.0) (Life Technologies).
- Ethidium bromide, 10 mg/ml (Life Technologies)
- Fluorescein avidin DCS (avidin-FITC, Vector), dilute stock solution (2 mg/ml in PNM) 1:100 with PNM. Store at 4°C.
- Fluorescein-12-dUTP, 1 mM (Roche Molecular Biochemicals). Store at -20°C.
- Formamide (FA, Life Technologies or Roche). Store at 4°C.
- Glycogen, 20 mg/ml (Roche Molecular Biochemicals). Store at -20°C.
- Geneclean II kit (BIO 101)
- Human COT1 DNA, 1 mg/ml (Life Technologies). Store at -20°C.
- Lambda phage DNA (Roche or Life Technologies). Prepare 2.5 ng/μl in 2×SSC. Store at 4°C.
- Low melting point agarose (Biorad)
- Lysozyme (Sigma Chemicals): prepare stock (50 mg/ml in 10 mM Tris, pH 7.5), and store in aliquots at -20°C. Do not refreeze.
- 10x PCR buffer: 15 mM $MgCl_2$, 100mM TrisHCl, 500 mM KCl, 0.01% gelatin.
- Phenol/Chloroform/Isoamyl alcohol: 25/24/1(vol./vol./vol.)(Life Technologies). Store at 4°C.
- Random-Priming Kit: BioPrime kit (Life Technologies). Store at -20°C.
- Proteinase K (Roche Molecular Biochemicals): 20 mg/ml in 10 mM TrisHCl, pH 7.5. Store at -20°C.

- RNase (Roche Molecular Biochemicals), DNase-free: boil at 100°C for 10 min., aliquot and store at -20°C
- Salmon sperm DNA (3'-5', Boulder, CO), 20 mg/ml. Store at -20°C.
- Sodium dodecyl sulfate (SDS)(Na salt, Sigma Chemicals): 10% in water.
- Thermus aquaticus (Tag) DNA polymerase, 5 U/µl (Perkin Elmer). Store at -20°C.
- Ultrapure water (Mallinckrodt, cat. # H453)
- Yeast artificial chromosome (YAC) library (Research Genetics). Store at -80°C.
- YOYO-1 (Molecular Probes): Stock is 1mM in DMSO. Dilute 1:1000 with water prior to use. Store at -20°C and discard diluted dye after 1 week.
- Zymolase (Sigma Chemicals, 70,000 U/g): prepare 10 mg/ml in 50 mM KH_2PO_4, pH 7.8, 50% glycerol. Store at -20°C.

Buffers and other solutions

- AHC medium (BIO 101): add 36.7 g of AHC powder per liter of purified water, autoclave at 121°C for 15 min.
- AHC agar (BIO 101): add 53.7 g of AHC agar medium per liter of purified water. Autoclave at 121°C for 15 min. Cool to 50°C. Then, mix well and pour plates. Store plates at 4°C.
- Alkaline Lysis (AL) solutions sufficient for 12 preps at the level of 20 ml cell culture:
  - AL Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0, add 4 ml of 0.5 M glucose, 0.8 ml of 0.5 M EDTA and 1 ml of 1 M Tris-HCl to 34.2 ml water. Store at 4°C.
  - AL Solution II: 0.2 N NaOH, 1% SDS. Add 1.4 ml of 10 N NaOH, 7 ml of 10% SDS to 61.6 ml water.
  - AL Solution III: 3 M NaOAc, pH 4.8
- Antifade solution: 1% p-phenylenediamine (Sigma), 15 mM NaCl, 1 mM H_2PO_4, pH 8.0,90% glycerol. Store at -80°C.
- Blocking stock solution: dissolve blocking reagent (Roche Molecular Biochemicals, cat. # 1096 176) in maleic acid buffer (10% w/v) with shaking and heating. Autoclave stock solution and store in aliquots at 4°C.

- Cell fixative: acetic acid/methanol, 1:3 (vol:vol.). Make fresh before use.
- DB 0.5 solution: 0.5 M EDTA (pH 8.0), 1.0% N-lauroyl sarcosine (Sigma), 0.5 mg/ml Proteinase K (Roche Molecular Biochemicals).
- Denaturing solution: 70% FA, 2xSSC, pH 7.0. Prepare fresh at least every 2 weeks. Store at 4°C.
- ES Buffer: 0.5 M EDTA (pH 8.0), 1% sarcosyl.
- Gel loading dye: 1% bromophenol blue in 30% glycerol.
- Hybridization Master Mix: 14.3% w/vol dextran sulfate, 78.6% FA, 2.9×SSC, pH 7.0. For 10 ml MM2.1, mix 1.45 ml of 20×SSC with 0.7 ml ultrapure water, dissolve 1.43 g dextran sulfate (Calbiochem), incubate overnight, then add 7.86 ml formamide. Aliquot in 1.5 ml microcentrifuge tubes and store at -20°C.
- Lysis buffer: 1% Triton X-100, 20 mM TrisHCl, 2 mM EDTA, pH 8.5.
- Maleic acid buffer: 100 mM maleic acid, 150 mM NaCl, adjust to pH 7.5 with concentrated NaOH
- Modified nucleotide mix (10x) for labeling in combination with 1 mM dig-11-dUTP or FITC-12-dUTP: combine 5 µl each of 100 mM dATP, 100 mM dGTP and 100 mM dCTP with 2.5 µl of 1M Tris-HCL, pH 7.5, 0.5 µl 0.5 M EDTA, pH 8.0 (Life Technologies) and 232 µl ultrapure water for a total of 250 µl. Store at -20°C. The final concentration of nucleoside triphosphates is 2 mM each.
- PNM: Dissolve 5 g of non-fat dry milk in 100 ml of PN buffer (PN buffer is 0.1 M sodium phosphate, pH 8.0, 0.1% nonidet-P40), incubate at 50°C overnight and add 1/50 vol. sodium azide, spin at 1000 g for 30 min, aliquot clear supernatant into 1.5-ml tubes and store at 4°C. Spin at 2000 g for 30 s prior to use.
- SCE: 1 M sorbitol, 0.1 M Na citrate, 10 mM EDTA, pH 7.8.
- Slide Blocking Solution (5×SSC containing 2% Blocking Reagent, 0.1% N-lauroyl sarcosine): combine 0.05 g N-lauroyl sarcosine (Na salt, Sigma) and 1 g Blocking Reagent with 12.5 ml of 20×SSC (pH 7.0), add 30 ml water, heat to 60°C while stirring and bring the final volume to 50 ml with ultrapure water, when the Blocking Reagent is dissolved. Aliquot into 1.5-ml tubes, spin at 2000 rpm for 10 min and store at 4°C.
- SSC: 20×SSC is 3 M NaCl, 0.3 M Na_3citrate × $2H_2O$, pH 7.0.

- 10× Taq buffer: 500 mM KCl, 100 mM Tris HCl, pH 8.3, 10 mM $MgCl_2$.
- TBE (Tris/borate/EDTA) buffer, 10× is 890 mM Tris base, 890 mM boric acid, 20 mM EDTA.
- TE (Tris/EDTA) buffer, 1× is 10 mM Tris HCl, 1 mM EDTA, pH 7.4, 7.5 or 8.0.
- TE 50 buffer: 10 mM Tris HCl, 50 mM EDTA, pH 7.8.
- Tris HCl [tris(hydroxymethyl)aminomethane]: 1 M, pH 7.5 or 8.0.

Subprotocol 1
Preparation of aminopropyltriethoxysilane (APS) derivatized slides

The derivatization of glass substrates is among the most critical steps of the procedure. The slides should have the capacity to bind DNA molecules at one or both ends, but allow the molecules to stretch during the subsequent drying.

Procedure

1. Slide preparation

1. Clean glass slides mechanically by repeated rubbing with wet cheesecloth to remove dust and glass particles.
2. Rinse several times with ultrapure water.
3. Immerse slides in boiling ultrapure water for 10 min.
4. Air dry.
5. Immerse slides in 18 M sulfuric acid for at least 30 min to remove organic residues.
6. Immerse in boiling water for 1-2 min.
7. Air dry and store until further use.

2. Silane modification

1. Immerse precleaned dry slides in a solution of 0.1% APS in 95% ethanol for 10 min.
2. Remove slides from the silane solution.
3. Rinse several times with water, and immerse in ultrapure water for 2 min.
4. Dehydrate by immersing in absolute ethanol.
5. Dry upright for 10 min at 65°C on a hot plate.
6. Store slides for 2-6 weeks at 4°C in a sealed box under nitrogen prior to use.

Coverslip silanation is performed similar to the procedure described for slides. Briefly, coverslips are rinsed with distilled water and dehydrated in 100% ethanol. Coverslips are derivatized with a 0.05-0.1% solution of APS in 95% ethanol for 2 min. Coverslips are then rinsed and dried as described above (steps 3-4).

Subprotocol 2
Preparation of high molecular weight (HMW) DNA

The YAC clones used in the examples presented here, were part of the CEPH/Genethon library, which is commercially distributed by Research Genetics. Other clones might be used in their place without changing the protocols. Information for many CEPH of the clones including insert size, STS contents and radiation hybrid or genetic map position is available from the CEPH/Genethon server at URL http://www.genethon.fr/genethon_en.html/ or the Massachusetts Institute of Technology (MIT) server (URL http://www-genome.wi.mit.edu/).

The agarose plug preparation and pulsed field gel electrophoresis using a CHEF electrophoresis system (BioRad) follow standard protocols. Typically, 5 to 15 individual YAC colonies are tested to account for deletions. In most cases, the largest clone carries the least deletion(s).

The P1/PAC/BAC clones typically show far fewer deletions, so that it often suffices to pick 2-3 colonies from a plate, grow the cells overnight, and extract the DNA using an alkaline lysis protocol. The DNA can then be loaded directly onto the PFGE gel using a common gel loading dye.

The DNA is recovered from the low melting point agarose slab gel by excising the appropriate band using a knife or razor blade. High molecular weight DNA is then isolated by β-agarase digestion of the gel slices.

Procedure

1. Pulsed field gel electrophoresis (PFGE)

Preparation of gel plugs containing YACs

1. Spin down cells from 5 ml AHC media at 400 rpm for 6 min. Resuspend cells in 0.5 ml of 0.125 M EDTA, pH 7.8. Spin again and remove supernatant.
2. Resuspend the ~ 70 µl cell pellet in 500 µl of SCE. Mix with an equal volume of 1.5% LMP agarose preheated to 43°C. Quickly pipette up and down, then vortex for 1-2 s to mix. Pipette into plug molds (Biorad) and allow to solidify at room temperature or on ice.
3. Remove plugs from molds, incubate samples in 2 ml SCE containing 100 µl of zymolase and shake at 150 rpm at 30°C for 2.5 h to overnight.
4. Remove SCE and add 2 ml of ES containing 100 µl of proteinase K (20 mg/ml). Shake 5 h to overnight at 50°C.
5. Remove ES and rinse 5 times with 6 ml of TE50 for 30 min each rinse. Store the plugs at 4°C.

PFGE running conditions

1. YACs: voltage gradient, 6 V/cm; switching time, 79 s initially, 94 s final; running time, 38 h; agarose concentration, 1.0% LMP agarose; running temperature, 14°C; running buffer, 0.5×TBE.
2. P1/PAC/BAC clones: voltage gradient, 6 V/cm; switching time, 2 s initially, 12 s final; running time, 18 h; agarose concentration, 1.0% LMP agarose; running temperature, 14°C; running buffer, 0.5×TBE.

For probe production and determination of optimal PFGE conditions: stain the gel with ethidium bromide (EB, 0.5 µg/ml in water), cut out a gel slice containing the target DNA band and transfer slice to a 14 ml polystyrene tube (Cat.# AS-2264, Applied Scientific). Wash slice with ultrapure water for 30 min, and then wash with 1× agarase buffer for 30 min.

For high molecular weight DNA isolation: run duplicate samples on the right and left side of the gel, respectively. After a predetermined run time, cut gel in half, and stain one half with EB. Measure the migrated distance

on a UV trans-illuminator, cut out a gel slice at the corresponding position from the unstained half and proceed as described in z.

2. Recovery of high molecular weight DNA from LMP agarose gel slices

1. Melt the gel completely by incubating it for 10 min at 85°C.
2. Transfer the molten agarose to a 43°C water bath.
3. Add 1 µl β-agarase for every 25 µl of molten agarose.
4. Incubate at 43°C for 2 h.
5. Add an equal volume of 200 mM NaCl.
6. Store the sample at 4°C until use.

3. Genomic DNA

Genomic high molecular weight (HMW) DNA is isolated from exponentially growing human cells such as the C32 melanoma cell line (ATCC) or diploid fibroblast cells using standard procedures. Briefly, about 5×10^5 cells are washed in PBS. The cells are then resuspended in 0.5 ml of PBS and mixed with 1.2% low melting point agarose previously melted in PBS and allowed to cool down to 43°C. Aliquots of 100µl are dispensed into plug molds and allowed to set for 30 min at 4°C. Agarose plugs are then placed into DB 0.5 solution and incubated overnight at 50°C. Next, plugs are washed 4-6 times for 30 min each in 50 mM Tris HCl, 1mM EDTA and stored at 4°C. The HMW DNA is released by digestion of the plugs with β-agarase according to the manufacturer's instructions (NEB) as described above.

Subprotocol 3
Immobilization and stretching of DNA molecules

The correct immobilization of DNA molecules is important for the successful stretching as well as the minimization of DNA loss during denaturation and hybridization. We have used different methods for binding and stretching of DNA on APS-pretreated surfaces. The quality of the resulting DNA fibers on glass or mica surfaces (Hu et al., 1996) appears to be

determined primarily by the DNA preparation and by properties of the modified surface rather than by the method of DNA stretching. In general, the ideal APS-surface binds the DNA molecules only at their ends, or in the case of circular DNA molecules, at the position of nicks. The remainder of the DNA molecule should be free in suspension. This can be observed in the fluorescence microscope after addition of 1 μM YOYO-1 to the DNA before immobilization.

Procedure

In a typical experiment, 1-2 μl of clonal or genomic DNA are mixed with an equal amount of YOYO-1 (1 μM or 0.1 μM) and 8 μl water. One or two microliter of this diluted DNA is applied to an untreated coverslip, which is then placed DNA side down on the APS-treated slide or coverslip. The DNA concentration can be estimated under the fluorescence microscope using a filter set for FITC, and adjusted as needed. As early as 2 min of incubation at room temperature, the untreated coverslip can be removed slowly from one end, allowing the receding meniscus to stretch the bound DNA molecules ('fibers') in one direction (Hu et al., 1996). Alternatively, the slide or coverslip sandwich can be allowed to dry overnight at room temperature, after which the untreated coverslip is removed. Slides or coverslips carrying DNA fibers are rinsed briefly with water, drained, allowed to dry at room temperature and 'aged' in ambient air at 20°C for 1 week before hybridization.

Subprotocol 4
Probes generated from cloned DNA fragments

A typical QDFM experiment uses several different probes simultaneously. One probe is needed to counterstain the DNA fibers. This probe is usually prepared by labeling DNA from the same batch that was used to prepare the fibers. Probes for sequences to be mapped along the fibers are made such that they can be detected in a different color. Furthermore, it is recommended to include landmark probes that provide reference points by binding specifically to the vector part or the ends of DNA molecules.

Procedure

1. Alkaline lysis protocol and purification of DNA from P1, PAC or BAC clones

This protocol describes the isolation of DNA from ~20 ml overnight cultures using 40 ml Oakridge centrifugation tubes. The protocol can be scaled down to accommodate smaller volumes.

1. Grow culture overnight in ~30 ml LB or TB medium containing the recommended amount of antibiotic.
2. Prepare Oakridge tubes. Write the clone ID on a small piece of tape stuck to the cap. Spin 18.5 ml of culture at 2000 *g* for 10 min at 4°C and discard the supernatant.
3. Resuspend the pellet in 2340 µl of AL Solution I, then add 100 µl of lysozyme stock to each tube. Incubate tubes for 5 min at room temperature. Then, place the tubes on ice.
4. Add 5.2 ml of AL Solution II. The mixture should now become clear. Mix gently by inverting the tubes several times. Incubate for 5 min on ice.
5. Add 3.8 ml of AL Solution III and mix gently by inverting the tubes several times. Incubate for 10 min on ice.
6. Spin for 15 min at high speed (11,500 rpm/14,000g).
7. Transfer 10.4 ml of supernatant into a new Oakridge tube, add 5.8 ml of isopropanol and mix gently by inverting the tubes several times. Use the old cap [with the ID sticker] on the new tube.
8. Spin for 5 min at ~10,000 *g* and discard the supernatant. Watch the pellet!
9. Wash the pellet in cold 70% ethanol. Let the pellets dry briefly, i.e., for ~20-40 min at 20°C to 37°C.
10. Resuspend the pellet in 0.8 ml of TE buffer and split the volume into two 1.5 ml microcentrifuge tubes.
11. Add 400 µl phenol/chloroform/isoamyl alcohol to each tube. All centrifugations during the following phenol/chloroform extraction are done at 12,000 *g*.
12. Vortex for 15 s and spin down for 3 min.

13. Remove most of the bottom layer and spin again for 3 min.
14. Transfer the top layer to new microcentrifuge tubes and add 400 µl chloroform/isoamyl alcohol (24:1, vol.vol.)
15. Vortex well for 15 s, spin down for 3 min and remove most of the bottom layer followed by a second centrifugation for 3 min.
16. Transfer top layer to a new microcentrifuge tube, add 2.5 volumes, i.e., 1 ml 100% ethanol and let the DNA precipitate for 30 min at -20°C.
17. Spin down for 15 min, discard the supernatant and wash the pellet in ice cold 70% ethanol, spin again briefly, remove supernatant and air dry the pellet.
18. Resuspend the pellet in 20-40 µl TE, pH 7.4 containing 10 µg/ml RNase.
19. Incubate 30 min at 37°C [in water bath]; then, store at -20°C until used.

2. Preparation of DNA from Yeast Artificial Chromosome (YAC) clones

Retrieve the desired yeast clone containing the YAC from the library and grow it on AHC agar for 2-3 days at 30°C. Pick colonies from the plate and culture the clones in up to 35 ml AHC media at 30°C for 2-3 days.

DNA extraction, phenol purification and alcohol precipitation

1. Centrifuge cells (in ~35 ml AHC media) at 2000g at 4°C for 5 min.
2. Decant the supernatant and resuspend cells in 3 ml total of 0.9 M sorbitol, 0.1M EDTA, pH 7.5, containing 4 µl β-mercaptoethanol, followed by addition of 100 µl of zymolase (2.5 mg/ml), and then incubate at 37°C for 60 min.
3. Pellet the cells at 2000 *g* and 4°C for 5 min and decant supernatant.
4. Resuspend pellet in 5 ml of 50 mM Tris, pH 7.4, 20 mM EDTA. Add 0.5 ml of 10% SDS and mix gently. Incubate at 65°C for 30 min.
5. Add 1.5 ml of 5 M potassium acetate and place on ice for 60 min.
6. Spin at 12,000 *g* for 15 min at 4°C, and transfer the supernatant to a new tube.
7. Mix the supernatant gently with 2 volumes of 100% ethanol by inverting the tube a few times. Spin at 5000 rpm (2000g) for 15 min at room temperature.

8. Prepare 1.5 ml microcentrifuge tubes (4 tubes per clone with ID on top).
9. Decant supernatant and air dry the pellet. Resuspend pellet in 3 ml of 1×TE, pH 7.5.
10. Transfer the DNA solution to four 1.5 ml microcentrifuge tubes.
11. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0), vortex well and spin at high speed (10,000 *g*) for 3 min.
12. Transfer the top layer to new 1.5 ml microcentrifuge tubes and add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex well and centrifuge at high speed (10,000 *g*) for 3 min.
13. Transfer the top layer to new 1.5 ml microcentrifuge tubes. Add 40 µl of RNase (1 mg/ml, DNAse free) to each of the four tubes and incubate at 37°C for 30 min.
14. Add 1 volume of isopropanol and gently mix by inversion. Centrifuge at high speed (10,000 *g*) for 20 min.
15. Decant supernatant and wash pellet with 1 volume of cold 70% ethanol, and centrifuge at high speed (10,000 *g*) for 3 min.
16. Decant the 70% ethanol and air dry the pellet.
17. Resuspend pellet in 30 µl 1×TE, and measure DNA concentration after the pellet is completely dissolved.

Subprotocol 5
Generation of probes by in vitro DNA amplification

In vitro DNA amplification using the polymerase chain reaction (PCR) is a very efficient method to synthesize probe DNA. It can be applied to amplify a particular DNA sequence, such as a part of the cloning vector, or with mixed-base primers to perform arbitrary amplification of virtually any sequence of interest. As illustrated in the following paragraphs, the former amplification can be applied to prepare DNA landmark probes, while the latter allows the preparation of probes to counterstain the fibers.

Procedure

1. Cloning vector-specific probes

The generation of P1/PAC-, BAC- and YAC-vector probe DNA takes advantage of the access to published vector sequences. PCR primers are typically designed to amplify fragments of 1100-1400 bp of vector sequence. Several such oligonucleotide pairs have been designed in several laboratories including ours and are used in either single pairs or combinations. The PCR usually follows standard conditions, i.e., a Tris-HCl buffer containing 1.5 mM $MgCl_2$ and 1 unit Taq DNA polymerase per 50 µl reaction is used, annealing temperatures range from 50°C to 60°C.

On the other hand, the YAC cloning vectors pJs97 and pJs98, cloned in plasmid vectors (BRL), can be used to prepare probes useful to determine the orientation of the YAC insert (Duell et al., 1997). For this purpose, plasmid DNA is extracted using the above alkaline lysis protocol or a commercial kit and labeled by random priming as described below.

2. Mixed base oligonucleotide primed PCR

The DNA probes for counterstaining the YAC DNA fibers are generated by mixed base oligonucleotide primed PCR (sometimes referred to as degenerate oligonucleotide primed PCR or 'DOP-PCR') (Cassel et al., 1997). An aliquot of the HMW DNA obtained by PFGE for fiber preparation is PCR amplified for a total of 42 cycles with oligonucleotide primers that anneal about every 200-800 nucleotides. In our preferred scheme, we use two different DNA amplification programs. Initially we perform a few manual PCR cycles using T7 DNA polymerase to extend the oligonucleotide primers at a relatively low temperature. Next, DNA copies prepared in these first cycles are amplified using the thermostable Taq DNA polymerase and a rapid thermal cycling scheme.

In the first amplification stage, T7 DNA polymerase ('Sequenase II', Amersham Pharmacia Biotech) is used in 5-7 cycles to extend the mixed base primer JUN1 (5-CCAAGCTTGCATGCGAATTCNNNNCAGG-3, N=ACGT) that is annealed at low temperature. Briefly, 2-3 µl of HMW DNA solution are removed from the bottom of each tube and PCR amplified using the following conditions: denaturation at 92°C for 3 min, primer annealing at 20°C for 2 min and extension at 37°C for 6 min. Sequenase must be added after each denaturation. (See Chapter 29 by Fung et al. for details.)

In the second amplification stage, 20 µl of the reaction product are resuspended in a 200 µl Taq amplification reaction buffer and amplified with primer JUN15 (5-CCCAAGCTTGCATGCGAATTC-3) with the following PCR conditions: denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72 °C for 2 min, repeated for 35 cycles. After precipitation of the PCR products in 1.2 vol of isopropanol, the products are resuspended in 30 µl of TE buffer. Subsequently, 1.5 µl of this solution is labeled in a 25 µl random priming reaction incorporating FITC-12-dUTP or biotinylated dNTPs.

Subprotocol 6
Probe labeling via random priming and hybridization

Labeling of DNA by random priming is a reliable method and, in our laboratory, is applied routinely to label DNA fragments from 100 bp to several hundred kb. The procedure involves an initial thermal denaturation of the DNA to allow the random oligonucleotides ('primers') to anneal. Thus, restriction or hydrolysis of large molecules is not necessary. Several companies now offer kits for random priming reactions. Slight differences exist with regard to enzyme activity, amount of random primers and cost per reaction.

Procedure

Measurement of DNA concentration

The concentration of PCR products can be estimated from the agarose gels run to confirm target amplification. If a sufficient amount of clonal or genomic DNA is available, one or two microliters can be used to accurately determine the concentration using Hoechst 33258 fluorometry using a TK100 fluorometer (Pharmacia).

Random priming

1. Add 250 ng of DNA to water to a final volume of 7 µl in a 0.5-ml microcentrifuge tube.
2. Boil DNA at 100°C for 5 min, then quickly chill on ice.

3. For labeling with either dig-dUTP or FITC-dUTP, add:
   - 2.5 µl 10× Modified Nucleotide Mixture
   - 3.25 µl 1 mM dTTP
   - 1.75 µl dig-11-dUTP or FITC-12-dUTP (1 mM, ROCHE MOLECULAR BIOCHEMICALS, #1093 088)
   - 10 µl 2.5× Random primers (BioPrime kit, Life Technologies part# YO1393)

   Note: For labeling the DNA with biotin, add 2.5 µl 10× dNTP mix provided with the BioPrime kit (containing biotin-14-dCTP), 5 µl water, and 10 µl 2.5× random primers).

4. Mix well, add 0.5 µl DNA polymerase I Klenow fragment (40 units/µl, Life Technologies, part #YO1396) and incubate in a water bath at 37°C for 60-120 min.
5. Add 2.5 µl of 10× stop buffer (Life Technologies, part #YO1107, part of the BioPrime kit).
6. Store probe at -20°C until used.

Subprotocol 7
Fluorescence in situ hybridization (FISH)

All hybridizations are carried out overnight at 37°C in a moist chamber. Fiber hybridizations include a comparatively low concentration of a biotin- or FITC-labeled DNA probe prepared from the high molecular weight DNA that is used to prepare the fibers. This counterstain highlights the otherwise invisible DNA fibers and allows competitive displacement by the probes to be mapped along the DNA fiber (Weier et al. 1995, Duell et al. 1997). Additionally, one or several cloning vector-specific probes are included to allow a determination of the orientation of the insert.

Procedure

The hybridization procedure is very similar to protocols used with metaphase spreads:

1. Hybridization mix: combine 1 µl of each probe, 1 µl of human COT1 DNA (optional), 1 µl of salmon or herring sperm DNA, and 7 µl of hybridization master mix.

2. Apply the hybridization mixture to the slide and coverslip.
3. Denature the slide at 88-92°C for 90 sec on a hot plate.
4. Transfer the slide to a moist chamber and incubate overnight at 37°C.

Washing and detection steps are not much different from protocols used for FISH in interphase and metaphase cells that have been described in detail (Weier et al. 1995, Duell et al. 1997, 1998; Wang et al. 1997):

1. After hybridization, wash the slide three times in 2×SSC at 20°C for 10 min each.
2. Incubated the slide with 100 µl PNM buffer or blocking stock solution under a plastic coverslip at 20°C for 5 min.
3. The slide is then incubated at room temperature for 30 min with 100 µl PNM buffer containing AMCA-avidin (Pharmacia), anti-digoxigenin-rhodamine (Roche Molecular Biochemicals) and a mouse antibody against FITC (DAKO).

   Note: If only two labels are used, i.e., biotin and digoxigenin, bound probes are detected with avidin-FITC DCS (Vector) and anti-digoxigenin-rhodamine, respectively.
4. The slide is washed two to three times in 2×SSC for 15 min each at 20°C with constant motion on a shaking platform.
5. If necessary, signals are amplified using a biotinylated antibody against avidin raised in goat (Vector) followed by another layer of AMCA-avidin, a Texas Red-labeled antibody against sheep raised in rabbit (Sigma) and a horse-anti-mouse antibody conjugated to FITC (Vector) (Wang et al. 1996).
6. The slide is mounted in 8 µl of DAPI (0.05 µg/ml in antifade solution) and covered by a 22×22 mm coverslip.

Subprotocol 8
Digital image acquisition and analysis

Although not a prerequisite for QDFM, digital image acquisition and computer-assisted analysis greatly facilitate the analysis of hybridization images. Since QDFM is based on simple measurements of distances between probe hybridization domains, the analysis can alternatively be per-

formed on images recorded on film and either printed or projected on a screen.

Procedure

Images are acquired using a standard fluorescence microscope (Zeiss Axioskop) equipped with 63×, 1.25 N.A. and 40×, 1.2 N.A. objectives, and a filter set for excitation and simultaneous observation of DAPI, Texas Red/rhodamine, FITC and CY5 fluorescence, respectively (Chroma-Technology). Current filters are capable of excitation in single bands centered around 360, 405, 490, 555, and 637 nm, and visualization in multiple bands in the vicinities of 460 nm (blue), 520 nm (green), 600 nm (red) and 680 nm (infrared). Images are collected using a CCD camera (Xilix, Hamamatsu or Photometrics) connected to a computer workstation (Weier et al., 1995).

For determination of map positions, interactive software is available for either Apple Macintosh, IBM/PC or SUN computers that allows the user to trace DNA fibers by drawing a segmented line and then calculates the length of the line in pixels (Wang et al. 1996; Duell et al. 1997). The pixel spacing is known from the microscope objective used in the experiment (use a 63× objective for molecules up to 100 kb, a 40× objective for larger molecules) and is converted into μm (or kb using the factor of 2.3 kb/μm). After measuring all relevant distances along the DNA fibers in triplicate, the results in the form of lists are imported into Microsoft Excel spreadsheets and used to calculate average values for each fiber and mean values and standard deviations for individual experiments.

Results

Solid substrates for QDFM are prepared in batches of 20-50 by derivatization of standard microscope slides, coverslips or sheets of mica with APS, which results in primary amino groups on the surface (Weier et al. 1995; Hu et al. 1996). For DNA fiber stretching, a solution of target DNA molecules onto which probes are to be mapped is placed on an untreated coverslip and spread by placing the coverslip upside-down on the APS-derivatized glass or mica surface. Binding of DNA to the substrate and the stretching effect can be monitored by staining the DNA with YOYO-1 prior to deposition. This also allows the rejection of batches of slides that bind DNA too tightly. Following DNA binding and stretch-

ing, the coverslips are removed, the slides are rinsed briefly with double-distilled water, air dried and stored at 4°C.

The DNAs from plasmid, cosmid, P1/PAC and BAC clones are isolated using an alkaline lysis protocol and inserts are sized by PFGE. Digestion of DNA with a rare cutting restriction enzyme produces linear high molecular weight DNA molecules, but the alkaline lysis procedure typically provides sufficient amounts of nicked circular or randomly broken DNA suitable for QDFM (Wang et al. 1996). In general, the DNA is loaded onto a 1.0% low melting point agarose gel and electrophoresed for about 15 h. The band containing the desired linear or circular DNA is then excised from the gel, and the gel slice is digested with agarase. Similarly, YAC DNA from various clones is purified by PFGE. The integrity of DNA molecules can be assessed by microscopic inspection of aliquots of DNA stained with 0.5 μM YOYO-1, before high molecular weight DNAs are used for DNA fiber or FISH probe preparation or stored at 4°C in 100 mM NaCl.

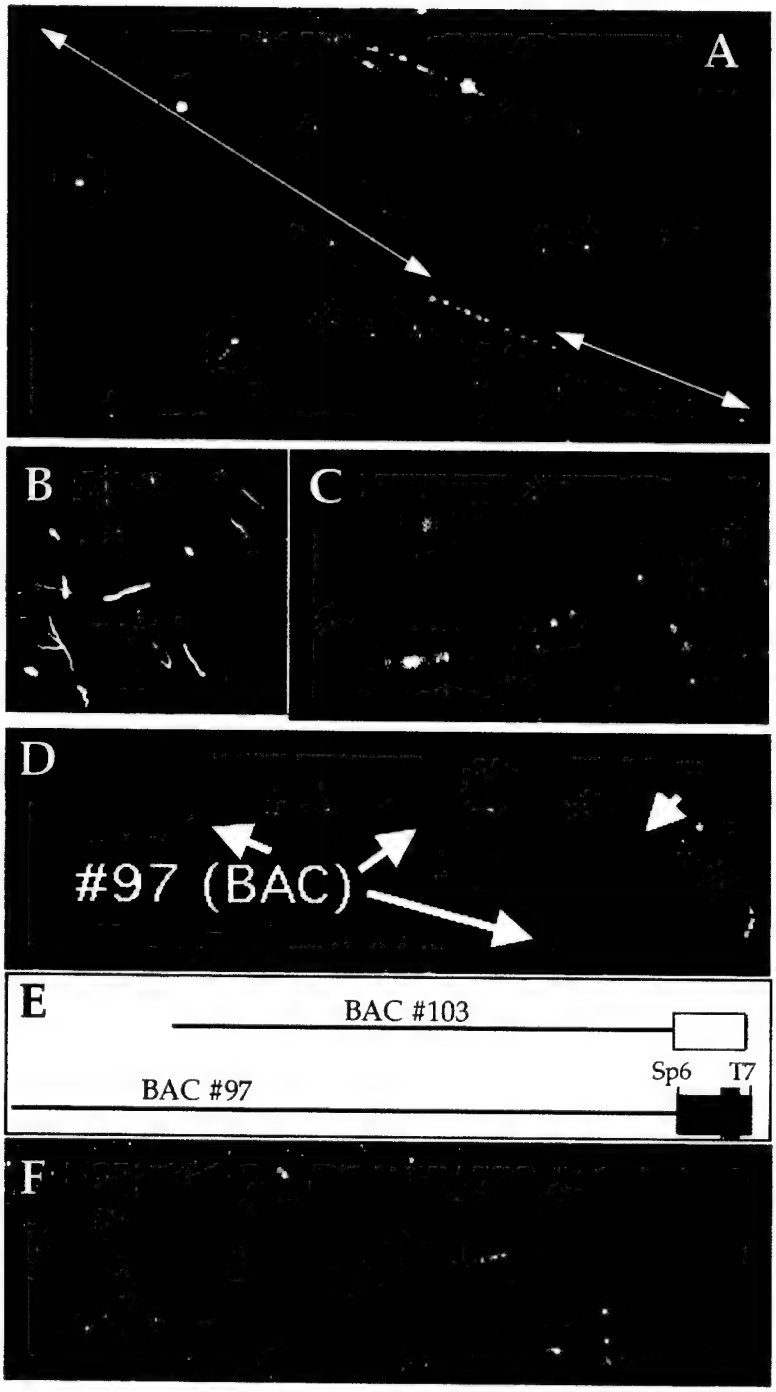
The density of DNA molecules after DNA fiber stretching can be adjusted by altering the concentration of the DNA molecules prior to binding. Figure 2 shows the typical density of hybridized lambda DNA molecules. In experiments depositing circular P1 and BAC DNA molecules, the fraction of intact DNA molecules sometimes reaches ~80%. While binding of DNA molecules in their circular form helps to maintain their integrity, it interferes with DNA fiber stretching, and the molecules are found to be stretched to varying degrees (Fig. 3B). Mapping onto circular molecules can thus be used for a rough estimation of overlap, and mapping on linear fibers for high precision measurements. This can be done in a single experiment, because some circular DNA molecules are sheared during deposition, thus providing randomly broken linear DNA molecules (Fig. 3B).

Quantitative DNA Fiber Mapping can facilitate the construction of high resolution physical maps comprised of any combination of cosmid, P1, PAC or BAC clones in two ways: if a low resolution map is available, for example, in the form of a YAC contig, individual clones can be mapped directly onto DNA fibers prepared from the larger clones (Weier et al. 1995, Cheng and Weier 1997). Alternatively, a high resolution map can be constructed by measuring the extent and orientation of overlap between individual clones (Fig. 1). In most experiments, the applied scheme will be determined by the sources of the clones and might combine both schemes. Figure 3 shows some typical examples of QDFM applications.

Figure 3A shows the mapping of a P1 clone (red) onto a colinear YAC clone (green). Precise localization of the region of overlap is facilitated by probes that mark specifically the ends of the YAC molecules (red).

Preparation and purification of the circular DNA molecules is simple and fast. An example of mapping an exon specific plasmid clone onto circular BAC molecules is depicted in Fig. 3C. The use of circular DNA molecules results in a dense deposition of circular DNA molecules in the presence of linear fragments of different sizes (Fig. 3B). The largest circles are stretched to about ~2.3 kb/μm, but smaller, more condensed molecules can also be analyzed using the extent of the vector-specific green ~7 kb domain on BACs as standard for normalization. The linear fragments

Fig. 2. Quantitative DNA Fiber Mapping (QDFM) using phage DNA molecules. Lambda DNA molecules immobilized on APS-derivatized glass slides were hybridized with a mixture of biotin- and digoxigenin-labeled lambda DNA restriction fragments. The molecules show specific labeling after incubation with avidin-FITC and rhodamine-labeled antibodies against digoxigenin. The *insert* shows a typical molecule


A
B
C
D
#97 (BAC)
E
BAC #103
Sp6
T7
BAC #97
F

found on the same slides are stretched more homogeneously, thus providing DNA fibers without need for normalization.

Expressed sequences can be mapped easily by QDFM, if each individual target extends for a few hundred base pairs or more. A common approach hybridizes small genomic DNA fragments of 1-2 kb that contain known exons onto larger genomic DNA molecules. If the cDNA sequence and some information about intron-exon boundaries are available, such small DNA fragments can rapidly be generated from genomic DNA using PCR. Figure 3C demonstrates this by mapping exon 2 of the human Band 4.1 gene onto a homologous BAC molecule. This allowed the localization of the ~2 kb exon with near kilobase precision.

Figures 3D,E illustrate the application of QDFM for measurement of BAC clone overlap. In such experiments, the DNA fiber is counterstained by a probe detected in blue. A BAC vector-specific probe (green) and a PCR-generated probe of ~1300 bp (red) that binds close to the T7 end of the vector are included to highlight the vector part of the DNA fibers (Figs. 3C,D). The two BAC clones shown in Fig. 3D overlap by approximately 80 kb and the overlapping region is close to the SP6 promoter in the BAC vector (Fig. 3D,E). These results are summarized schematically in Fig. 3E.

A rapid approach to studying the genomic organization of genes relies on direct mapping of expressed sequences. The probe DNA is isolated from cDNA clones, labeled and hybridized onto genomic DNA fibers. In the presence of blocking DNA, the cDNA probes will bind specifically to their complementary DNA targets, i.e., exons and 5- or 3-untranslated regions (UTRs) along the DNA fiber. This leaves non-coding regions (introns, 5- and 3-flanking DNA) unstained (Fig. 3F). Using FISH conditions similar to those

Fig. 3. Quantitative DNA Fiber Mapping (QDFM) using large insert human genomic DNA clones. **A** Mapping P1 clones along YAC molecules. The *arrows* indicate the distance from the respective ends. **B** Circular DNA molecules excised from a PFGE gel purified and stained with YOYO-1 revealed closed circular DNA molecules in the presence of linear molecules of different length. **C** Physical mapping of small cDNA clones in larger genomic intervals. DNA fibers (blue) prepared from a BAC clone were hybridized with a ~2 kb insert of a plasmid containing exon 2 of the human Band 4.1 gene (red). **D, E** Determination of overlap between linked BAC clones. A probe prepared from BAC # 103 (red) was hybridized onto DNA fibers prepared from BAC #97 (blue). **E** shows a schematic representation. **D** Mapping the genomic organization of expressed sequences. Here, BAC DNA fibers (blue) were hybridized with a ~5 kb cDNA probe (red). Three hybridization domains representing larger exons and the 3 UTR were detected on the DNA fibers. The green probe in **C, D** and **F** delineates the BAC vector

applied to hybridization and probe detection on metaphase chromosomes, this system works well with cDNA probes of several kb.

Troubleshooting

- **Slide pretreatment**
  Slides from different manufacturers or even of the same brand may produce very different qualities of fibers. Use a large batch of slides from one manufacturer; avoid slides that are painted on one end, since the paint might come off during pretreatment. Slides that have a sand-blasted area at one end are preferable.

- **Homogenous stretching of DNA molecules**
  Different procedures have been described to stretch DNA molecules. In our hands, stretching involving a hydrodynamical force (meniscus) at 20° C or 4° C has proven most reproducible. There is, however, no need to wait until the preparation has dried to completion. Once the DNA molecules have bound to the substrate, the coverslip can be lifted to exert the hydrodynamic stretching force (Hu et al. 1996).

- **Immunocytochemical signal amplification**
  Never let the slides or part of them dry out during the immunocytochemical signal amplification. It is important to just drain the liquids from the slides, and then rapidly apply the next solution such as a blocking solution or the antibodies. If the slides are allowed to dry out, the level of background staining will increase to unacceptable levels.

- **Image acquisition**
  Most fluorochromes fade very quickly. Thus, minimize the exposure of slides to the excitation light. Don't waste time studying the beauty of the molecules.

- **Image analysis**
  Always measure additional segments of the molecule such as the vector segment, since these might provide additional information about the extent and homogeneity of DNA stretching.

- **Errors**
  Relative standard deviations are typically in the order of 5%. Higher standard deviations provide a simple means of controlling the procedure. They signal it is necessary to check the data analysis results for

operator errors and undesirable images such as broken molecules or insufficiently stretched fibers.

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Suppliers

5 Prime -3 Prime, 5603 Arapahoe, Boulder, CO 80303, USA

Amersham Pharmacia Biotech, Amersham Pharmacia Biotech, Inc., 800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327, USA; phone 1-732-457-8000, fax: 1-732-457-0557; http://www.amersham.com.au/

Applied Spectral Imaging Inc., 2120 Las Palmas Drive, Suite D, Carlsbad, CA 92009, USA; phone (800) 611-3466, fax (760)929-2842

BIO-RAD Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547-9980, USA; phone (510) 741-1000, fax (510) 741-5800, http://www.bio-rad.-com/index1.html

Calbiochem, 10394 Pacific Center Court, San Diego, CA 92121 USA; phone (800) 854-3417, fax (800)776-0999

Fisher Scientific, 9999 Veterans Memorial Drive, Houston, TX 77038-2499, USA; phone (800) 766-7000, fax (800) 926-1166

Life Technologies, P.O. Box 9418, Gaithersburg, MD 20898, USA; phone (301) 840-8000, fax (301) 670-8539

International Equipment Company (IEC), 300 Second Avenue, Needham Heights, MA 02194, USA, phone (617) 449-8060, fax (617) 444-6743

Labline Instruments, 15th and Bloomingdale Ave., Melrose Park, IL 60160-1491, USA; phone (800) 522-5463, fax (708) 450-0943, http://www.labline.-com, email: value@labline.com

New Brunswick Co., Inc., 44 Talmadge Rd., Edison, NJ 08818-4005, USA; phone (908) 287 1200, fax (908) 287 4222, email bioinfo@nbsc.com, http://www.nbsc.com

New England Biolabs, Inc., 32 Tozer Road, Beverly, MA 01915-5599, USA; phone (800) 632-5227, fax (978) 921-1350, http://www.neb.com

Nikon, Inc., 1181 Chess Drive, Suite H, Foster City, CA 94404-1109, USA; phone (650) 513-0542, fax (650)513-0540

Perkin Elmer, 850 Lincoln Center Drive, Foster City, CA 94404, USA; phone (800) 345-5224, fax (415)572-2743

Pharmacia Biotech Inc., 800 Centennial Ave., P.O. Box 1327 Piscataway, NJ 08855-1327, USA; phone (800) 526 3593, fax (800) 329 3593, http://www.biotech.pharmacia.com

Research Genetics, Inc., 2130 Memorial Pkwy, SW, Huntsville, AL 35801, USA; phone (205) 533 4363, fax (205) 536 9016, http://www.resgen.com/

Roche Molecular Biochemicals, 9115 Hague Rd., P.O. Box 50414, Indianapolis, IN 46250-0414, USA; phone (800) 428-5433; fax (800) 428-5433

Sigma, P.O. Box 14508, St. Louis, MO 63178, USA; phone (800) 325-3010, fax (800)325-5052

Thermatron Industries, Inc., 291 Kollen Park Drive, Holland, MI49423, USA; phone (616) 393-4580, fax (616)392-5643

Vector Laboratories, Inc., 30 Ingold Road, Burlingame, CA 94010, USA; phone (415) 697-3600, fax (415)697-0339

VWR Scientific, 1310 Goshen Pkwy., West Chester, PA 19380, USA; phone (800)932-5000, fax (610) 436-1761; email solutions@vwrsp.com; http://www.vwrsp.com/

Vysis, Inc., 3100 Woodcreek Drive, Downers Grove, IL, USA: phone (800) 553-7042, fax (630) 271-7138

Abbreviations

AFM	atomic force microscopy
BAC	bacterial artificial chromosome
CEPH	Centre des Études du Polymorphisms Humain, Paris, France
LMP	low melting point
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
QDFM	quantitative DNA fiber mapping
UTR	untranslated region
YAC	yeast artificial chromosome

FISH in cancer diagnosis and prognostication: from cause to course of disease

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The last 20 years have witnessed an astounding evolution of cytogenetic approaches to cancer diagnosis and prognostication. Molecular techniques and, in particular, nonisotopically-labeled nucleic acid probes and fluorescence *in situ* hybridization (FISH)-based techniques have replaced the costly and potentially dangerous radioactive techniques used in research and the clinical detection of genetic alterations in tumor cells. Fluorescent DNA probes also enabled the screening for very subtle chromosomal changes. Clinical laboratories now choose from a growing number of FISH-based cytogenetic tests to support physician's diagnoses of the causes and the course of a disease. Depending on the specimen, state-of-the-art FISH techniques allow the localization and scoring of 10–24 different targets and overcome previous problems associated with target colocalization and detection system bandwidth. FISH-based analyses have been applied very successfully to the analysis of single cells and have demonstrated the existence of cell clones of different chromosomal make-up within human tumors. This information provides disease-specific information to the attending physician and should enable the design of patient-specific protocols for disease intervention.

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Most tumors are comprised of cells that escape the tightly regulated cellular life-cycle of generation, differentiation, senescence and death. Typically, genetic changes accumulate during neoplastic development and provide tumor cells with unusual growth and proliferative characteristics, most of which are inscribed in the heritable genetic code of the cells. Often referred to as mutations, these heritable changes provide genetic markers by which tumor cells might be identified and differentiated from surrounding normal cells. However, a precise determination of a normal or abnormal genetic make-up of cells often proved difficult because cells were found in different stages of the cellular growth cycle or changes were so subtle that only very few nucleic acid bases were involved.

As we learn more about the genetic changes underlying neoplastic deviation, we are also challenged to develop technologies to rapidly identify cells presenting disease-specific markers. Fluorescence *in situ* hybridization (FISH) is a nonisotopical method to visualize the location of nucleic acid sequences in cells, cell organelles, tissue sections or whole mounts of small animals. As shown schematically in FIGURE 1, FISH is based on the preparation of a nucleic acid probe labeled with a fluorescent or immunogenic hapten followed by hybridization of the probe to a DNA target rendered single-stranded by denaturation. The most simple and thus, most common way of denaturing double-stranded nucleic acids is by heating the substance above the temperature required to break the hydrogen bonds that hold together the double helix. The probe is then allowed to anneal to its complimentary nucleic acid target sequence for up to several hours and unbound probe molecules are removed by stringent washes. Most fluorochrome-labeled probes can then be seen

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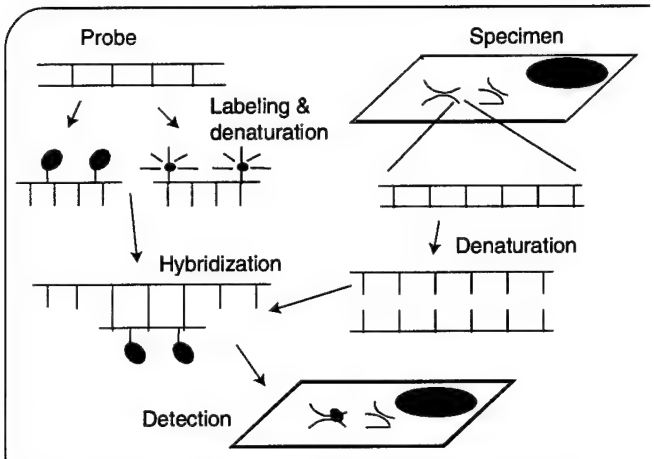

Figure 1. Schematic representation of the fluorescence *in situ* hybridization (FISH) procedure (See text for details).

directly in a fluorescence microscope. The detection of probes labeled with nonfluorescent haptens, such a biotin, digoxigenin or dinitrophenyl (DNP) typically involves the application of antibodies or affinity reagents, such as avidin or streptavidin, which carry fluorochromes for detection.

In the subsequent sections of this review, we will describe FISH applications on:

- Detection of specific numerical chromosome aberrations in tumor cells to aid diagnosis and tumor staging, to provide guidance in therapeutic decisions as well as to detect minimal residual disease
- Detection of specific structural chromosome aberrations associated with tumor development
- Genome-wide screening for numerical and structural chromosome aberrations to identify novel genes involved in the onset and progression of tumors

State-of-the-art FISH technology

Today's broad acceptance of FISH technology is a consequence of more than a decade of progress in two important areas. First, a vastly increased number of DNA probes and probes combinations are now available to aid researchers and clinicians in specific diagnostic investigations [1,2]. Second, innovative probe labeling techniques and significant advances in optical microscopy have helped to evolve FISH to the point where between 12–24 different DNA targets can be analyzed simultaneously on a routine basis [3–5]. The following paragraphs summarize the recent developments and highlight applications.

DNA repeat probes

Early applications of FISH involved the use of DNA probes that bind specifically to highly repeated DNA segments, such as the extensive arrays of satellite DNA found at the chromosomal centromeres and other heterochromatic regions [6]. The typical chromosome-specific target would be a large number of almost identical copies of the DNA repeat arranged in tandem [6,7]. Often, a single, small probe sequence is sufficient to label extensive parts of a chromosome as shown for the detection of the Y chromosomes in different species using probes of about 100–200 bp [8–10]. Strong signals are generated when the small, identical probe molecules find hundreds or even thousands of binding sites in close proximity. This also allows the detection of highly iterated tandem repeats using synthetic oligonucleotides, thus circumventing time-consuming molecular cloning, DNA preparation and labeling steps. If the probe and target are distinctly different from the rest of the genome and cross-hybridization is neglectible, probes can be applied at a vast molar excess over target sites and hybridization times may be as short as a few minutes.

Locus-specific DNA probes

The term 'locus-specific DNA probe' (LSP) refers to a single probe molecule or collection of labeled nucleic acid fragments that bind to a limited, single copy region of the genome. This typically contains a gene, a translocation breakpoint or another relevant DNA sequence. Target sizes of LSPs range from about 14 kb for lambda phage-derived probes [11] to more than a million basepairs (1 Mbp) for probes prepared from large yeast artificial chromosomes (YACs) [11–13]. LSPs have gained importance in the analysis of inter- or intrachromosomal rearrangements as well as the detection of small terminal deletions [13–16]. Access to LSPs is provided through commercial sources, such as Vysis Inc., Downers Grove, IL, USA, which offers a range of probes. These include numerous probes for known oncogenes or common translocations breakpoints. While not-for-profit organizations, such as the laboratories of Drs M Simon and J Korenberg in Los Angeles prepared extensive panels of physically mapped probes and make them available to the scientific community at minimal cost [17–20], companies, such as Research Genetics of Huntsville, AL and Children's Hospital Oakland Research Institute (CHORI, Oakland CA 94609, USA) offer a fee-for-service to screen large size insert genomic DNA libraries for clones containing a gene or sequence of interest [101–110]. As demonstrated below, multicolor FISH with LSPs allows assessment of the frequency of calls carrying specific aberrations known to be associated with tumorigenesis, analysis of the series of genetic changes that occur during tumor evolution and correlation between genotype and phenotype.

Whole chromosome painting probes

Whole chromosome painting (WCP) probes are collections of labeled nucleic acid fragments that have sequence homology with regions of the genome distributed over an entire chromosome or parts thereof. These probes are typically composed of a large number of different sequences and increasing the probe complexity, i.e., the fraction of the target that is represented by probe molecules, leads to more homogeneous staining. Different strategies including chromosome enrichment by fluorescence-activated chromosome sorting (FACS), interspersed repeat sequence (IRS) or arbitrarily-primed DNA amplification and microdissection have been applied to generate WCPs [21,22]. The main application of WCPs is the detection of trans-

locations involving nonhomologous chromosomes. WCPs are now commercially available for all human and mouse chromosomes and probes labeled with different fluorochromes can be combined to delineate as many as 24 different chromosomes. This technique, termed 'multiplex-fluorescence *in situ* hybridization' (M-FISH) [23] or spectral karyotyping (SKY) [3] will be discussed in the next chapter.

Recent advances in probe labeling & detection techniques

Labeling of nucleic acid probes has never been easier. Various companies offer kits that allow even the novice to label the DNA probes used in the planned FISH experiments. The reporter molecules of choice are fluorochromes, which if bound in sufficient quantity and density, can be observed in the fluorescence microscope without further signal amplification. In the early days of FISH, most probe DNA was labeled enzymatically by either random priming or nick translation. Today, a researcher planning to prepare his or her own probes can chose between enzymatical or chemical labeling techniques to modify the DNA [24,25].

Many projects, using such techniques as M-FISH [23] or SKY [3], require the use of relatively large amounts of multiple fluor- or hapten-labeled nucleotides for the preparation of DNA probes. Such a requirement makes these experimental approaches very expensive but the cost of such nucleotides can be reduced significantly by purchasing the chemical precursors, fluor or hapten succinimidyl esters and 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (AA-dUTP) and performing simple coupling and purification reactions [26].

The way in which DNA probes are labeled has changed and the modalities of their detection have also undergone major evolution. Early probe detection systems involved nonfluorescent haptens and some kind of antibody sandwiching technique to couple fluorescent molecules to the hybridized DNA probes [21]. While these detection schemes are still widely used, novel signal amplification schemes, such as rolling circle amplification [27], have been developed to boost the fluorescence signals so that even faint signals can be detected with ease. Another very efficient technique involves the binding of an enzyme complex to the hybridized probe followed by precipitation of a fluorescent substrate [28,29]. Under normal conditions, either amplification system increases signal strength by a factor of 10–30, allowing the detection of very faint signals derived from small DNA targets [30].

An increasing number of fluorescent reporter molecules can be identified by virtue of their fluorescence emission spectrum leading to the development of a variety of multicolor schemes for the detection of numerical and structural chromosome aberrations. Some groups use optical filter-based microscope detection systems [21,31–34] for multicolor FISH to excite one fluorochrome at a time. Other laboratories [3,35,36] favor a system in which a multicolor filter or mirror provides the light for the simultaneous excitation of several fluorochromes and the emission filter is replaced by an optical interferometer (FIGURE 2). This spectral imaging system combines

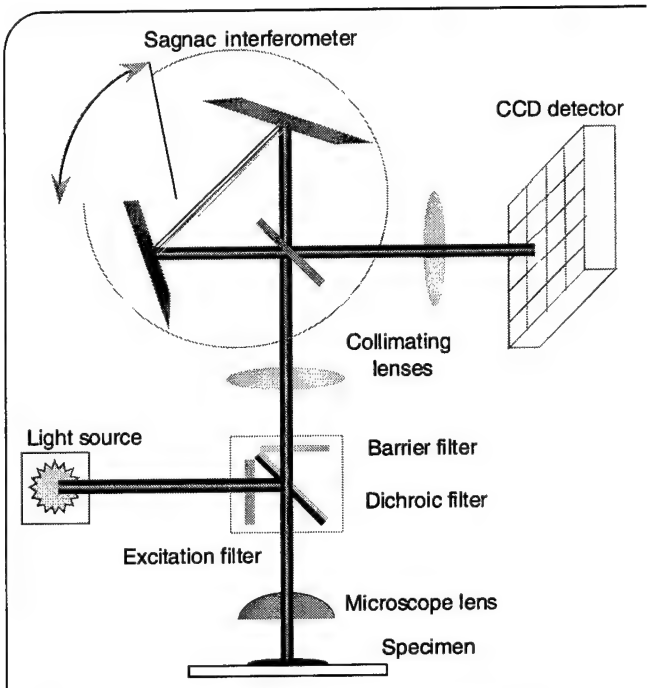

Figure 2. Schematic diagram of the optical pathway of the SpectraCube System (Image courtesy of Applied Spectral Imaging Ltd., Migdal Ha'Ermek, Israel).

the techniques of fluorescence optical microscopy, charged coupled device imaging, Fourier spectroscopy and software for digital image analysis. The power of this technology has been demonstrated by specific staining of all 24 human chromosomes in metaphase spreads, termed SKY [3,37].

The recent work of Fung *et al.* extended the application of spectral imaging to the cytogenetic analysis of interphase cell nuclei [4,35]. Five to six primary reporter molecules and a coloring scheme in which chromosome-specific probes are identified by the relative amount of each reporter molecule (termed ratio labeling) allowed scoring of up to 12 different chromosomes in a variety of interphase cell types [35]. The number of targets that can be accurately scored at the same time appears to be limited not by the labeling scheme but by the procedures available to homogeneously spread all the DNA so that spatial overlap of hybridization domains is minimized. Developed primarily for the analysis of single cells in *in vitro* fertilization and prenatal diagnosis, this technology should be easily adaptable to applications in tumor diagnosis.

Selected applications of FISH in cancer research & detection of minimal residual disease

Extra chromosomes as hallmarks of tumor cells

Trisomy 8 is observed in a significant fraction of patients with chronic myeloid leukemia (CML) [38]. In these cases, the presence of the extra chromosome 8 allows easy identification of the tumor cells and assessment of their frequency in mixed cell populations [39]. This information may become diagnostically useful and guide further therapeutic decisions. During therapy of leukemic diseases, for example, success of a specific treatment

regimen may depend on detecting early changes in the relative fraction of malignant cells in blood or bone marrow samples. FISH analysis with chromosome-specific DNA probes facilitates the differentiation between normal diploid cells and aneuploid tumor cells *via* the analysis of uncultured interphase cells. This not only reduces the time and effort required to prepare metaphase spreads for banding analysis but it also minimizes selection artifacts that might occur during cell culture [40,41]. FIGURE 3A illustrates the detection of tumor cells carrying an extra chromosome 8 among diploid bone marrow cells from a CML patient with a (47, XY, +8) tumor karyotype. Tumor cells can be identified rapidly by the presence of three hybridization signals per nucleus (FIGURE 3A, arrow).

Numerical chromosome aberrations are not limited to tumors of the hematopoietic system and they are found in a variety of solid tumors, among them renal cell cancers [42]. Metaphase spreads from these tumor cells are often difficult to obtain, thus preventing tumor studies by conventional G-banding analyses. The application of DNA repeat probes is often the method of choice for analysis of these tumors [43] and identification of tumor cells. FIGURES 3B–C show the example of interphase cells from a kidney tumor. Apparently, tumor cells are either near diploid or near tetraploid with extra copies of chromosome 8 (FIGURE 3C).

Detection of structural alterations in interphase cells

Structural chromosome alterations without gains or loss of DNA, such as reciprocal translocations, might alter the expression of oncogenes or tumor suppressor genes thus confer growth advantages to tumor cells. FISH with locus-specific probes that flank or span the breakpoint region are powerful tools to detect tumor cells as demonstrated for the bcr/abl translocation in CML [44,45]. FIGURE 3D–J illustrates the two complementary approaches. In this figure and all remaining figures, square marks next to a hybridization signal denote a red signal and triangular solid marks denote green signals. Circular open marks denote centrometic marker signals and arrows denote hybrid (red/green) signals on the same chromosome that cannot be distinguished with black and white images. Probes that span the breakpoint region can be labeled in different, i.e., chromosome-specific colors and hybridized in combination with DNA repeat probes (FIGURE 3D). As shown in (FIGURE 3F–G, cells from an individual carrying a balanced translocation t(3;4) can be analyzed for the presence and number of normal and derivative chromosomes. The probes prepared from yeast artificial chromosomes (YAC's) carry large inserts of human genomic DNA [12,13,46,47] and appear as single color hybridization domains after hybridization to normal chromosomes (FIGURE 3E and domains marked by arrowheads in FIGURE 3F). Derivative chromosomes, on the other hand, produce domains of closely spaced red and green fluorescence (arrows in FIGURE 3F,G) which can be distinguished easily from the single color domains associated with nonrearranged loci. The inclusion of one centromeric probe (marked with and open circle in this example) allows unambiguous identification of the derivative chromosomes [48]. Besides the identification of structural chromosome aberrations, hybridization of

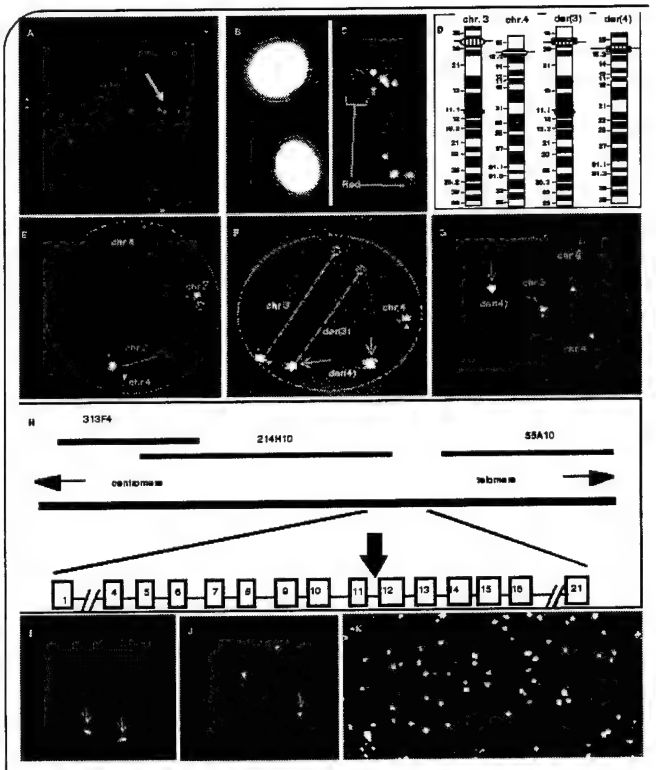

Figure 3. Locus-specific probes (LSP) detect chromosomal aberrations in interphase cells: (A) A chromosome 8–specific α satellite DNA probe identifies tumor cells in bone marrow aspirates from a leukemia patient with a tumor cell karyotype of (47,XY,+8) (arrow); **(B:** DAPI image, **C:** FISH image) In **(C)** combined hybridization of probes for chromosomes 8 (red; 5 spots indicated with lines) and 12 (green; all other spots) demonstrates the presence of cell clones of with extra copies of chromosomes 8 in interphase tumor cells in this case of a patient with kidney tumor; **(D)** A simplified hybridization scheme to detect structural and numerical chromosome alterations with probes spanning translocation breakpoints. A green probe spans a breakpoint region on chromosome 3 (oval with vertical stripes) and a probe detected in red spans the breakpoint on chromosome 4 (oval with horizontal strips). While normal chromosomes cause single color hybridization domains in interphase cells, the derivative chromosomes produce hybridization domains with adjacent red and green fluorescence (two closely adjacent ovals). Blue probes mark the centromeres of chromosome 3 thus allowing an accurate enumeration of the different chromosome types (stippled ovals); **(E)** Hybridization of locus-specific probes for chromosomes 3 and 4 to normal interphase cells produces large, single color domains representing the respective targets on chromosome 3 (green, square) and 4 (red, triangle), respectively; **(F)** Hybridization of the probes shown in **(D)** to cells derived from an individual carrying a reciprocal translocation t(3;4) shows single-color 'normal' domains (red, triangle; green, square) and the presence of translocation chromosomes in form of associated red-green domains (arrows); **(G)** Numerical as well as structural chromosome aberrations can be detected with locus-specific probes. Here, hybridization of the chromosome 3/4 probe set to embryonic cells demonstrates one copy of chromosome 3 (square), two copies of chromosome 4 (triangles) and one copy of a der(3) chromosome (arrow), i.e., aneusomy with respect to the distal parts of chromosomes 3p and 4p; **(H)** Hybridization scheme to detect ret gene rearrangements in interphase cells. Green-labeled probes binds to the 5'-end of the ret gene (313F4, 214H10), while a red labeled probes binds to the 3'-end of the gene (55A10). The common breakpoint maps into intron 11 of the ret gene. **(I)** Hybridization of the ret-specific probes to interphase cell nuclei from normal donors reveals two-hybridization domains with associated red-green staining (arrows); **(J)** Hybridization of the ret-specific probes to interphase cell nuclei from the thyroid cancer cell line TPC-1 ret gene shows one red-green domain representing a nonrearranged of the ret gene (arrow) and separated green and red domains (red, triangle; green, square) indicating the rearranged of the second homologue; **(K)** Amplification of the c-myc gene in a case of melanoma. A touching imprint preparation was hybridized with a probe specific for c-myc (red, a center cell marked with squares at green signals), which maps to 8q24 and a chromosome 8–specific centromeric DNA repeat probe as control (Vysis CEP 8, green, a center cell marked with triangles at red signals). Generally, the green signals are bright and fat; the red signals are smaller and less intense. (Image courtesy of J. Utikal, Department of Dermatology, University of Ulm, Ulm, Germany.) (see on-line version for color figures).

breakpoint spanning probes also allows the detection of numerical aberrations or aneuploidies [12,13]. In the example presented in FIGURE 3G, the reciprocal translocation in the father's germline interfered with the proper segregation of chromosomes in meiosis and led to aneuploid offspring [13,48]. In this case, hybridization involving breakpoint spanning probes as well as a chromosome enumerator (centromeric) probes helped to detect partial aneusomy in the embryonic cells [48]. The very same scheme can be applied to characterize cells from carriers of the translocation t(11;22), which is the most common translocation in humans and indicates an increased tumor risk in carriers [49].

The detection of structural rearrangements in tumor interphase cells with probes that flank one or both translocation breakpoints was first described for the above mentioned bcr/abl translocation involving genes on human chromosomes 9 and 22 [2,45]. FIGURE 3H shows a similar scheme developed to detect translocations that activate the ret proto-oncogene in papillary thyroid cancer (PTC) [50]. The common breakpoint in ret-activating translocations in PTC maps to intron 11 just upstream of the catalytic domain of this tyrosine kinase. As outlined in FIGURE 3H, we prepared probes that bind to either the 5′-end (clones 313F4 and 214H10) or the 3′-end of the ret gene (clone 55A10) and detected the bound probes with green or red fluorochromes, respectively. Mononuclear white blood cells from normal donors display the expected red/green domains (FIGURE 3I, arrows), whereas cells from the PTC cell line TPC-1 show one red/green 'normal' domain (arrow) and one set of separated red and green hybridization domains (FIGURE 3J). This was expected since TPC-1 is known to carry a rearranged copy of the ret gene [50].

Application of FISH in tumor prognostication

Numerous studies have shown that tumor development is accompanied by at least two changes:

- A change in the way cells interact with their environment *via* membrane-bound receptors
- A change in how signals originating from these receptors are transduced from the cell membrane to the cytoplam and the cell nucleus where it will alter the levels of expression of particular genes

Among the hundreds of genes involved in receptor-mediated signal transduction, only a few have been shown to be aberrantly expressed in tumors. For example, overexpression of tyrosine kinase genes, due to gene amplification or changes in the regulation of gene expression, may lead to oncogenic transformation. This has been clearly documented for the erbB-2 protein, the product of the Her-2/neu proto-oncogene and other members of the erbB family, especially in breast cancer patients. In addition, many tumors have acquired structurally altered tk proteins as well as an abnormal expression pattern through *de novo* mutational events. In cases where chromosomes have become rearranged, the catalytic domain of a tk gene can be fused to the amino terminal of another protein, thus creating a new, transforming activity as well as a new expression pattern. The above-mentioned activating ret rearrangement is just one example. Other well known examples of this mechanism of oncogene activation are the previously discussed bcr/abl-fusion protein in CML with t(9;22) and the activation of the receptor tyrosine kinase trk in papillary thyroid cancer.

Overexpression of particular genes, such as the insulin-like growth factor receptors (IGF-IRs), the epidermal growth factor receptor (EGFR or erbB) family of receptors, focal adhesion kinase (FAK) or the proto-oncogenes ret and Nyk/mer have been shown to correlate with progression to a more malignant phenotype in a variety of tumors. Other genes that have often been found amplified in solid tumors appear to be related to increased gene expression, such as the c-myc gene which maps to the long arm of human chromosome 8. Interphase FISH with locus-specific probes is able to accurately determine the number of copies of a gene per cell. Such detailed knowledge about gene amplification coupled with additional measurement of gene expression might increase our understanding of how tumors grow. Such knowledge potentially leads to the design of assays, which will allow us to perform more accurate staging of tumors and predict the course of tumor development, i.e., its capacity to grow, invade and spread to other sites.

As an example, FIGURE 3K illustrates the detection of gene amplifications in skin cancers. Tumor cells were transferred from a melanoma sample onto glass microscope slides by the touching imprint method and hybridized with a probe specific for the c-myc proto-oncogene (signals in FIGURE 3K a center cell marked with triangles at signals). A commercial probe that stains repeated DNA in the centromeric region of chromosome 8 (CEP 8, Vysis, Inc., labeled with green fluorochromes, a center cell marked with squares at green signals) was included to determine the number of whole chromosomes 8. Hybridization results (FIGURE 3K) confirm high level amplification of the c-myc gene without extra copies of chromosome 8 in these cells. This patient-specific information combined with results of population-based retrospective studies correlating c-myc gene amplification with tumor metastasis and average time of disease-free survival might allow the attending dermatologist/oncologist to prepare a more individualized therapy regimen [51].

WCP in the analysis of metaphase spreads

Whole chromosome painting (WCP) is a rapid technique to detect translocations involving nonhomologous chromosomes. High quality painting probes delineate the target chromosome from one end to the other, while the hybridization of nonspecific repeats, such as interspersed repeats or centromeric clusters of satellite DNA is blocked by addition of an excess of unlabeled repeat DNA to the hybridization mixture [22].

Simple chromosome painting experiments use only one or two WCP probes as shown in FIGURE 4A for the delineation of chromosomes 1 (square) or 8 (triangle). This is often cost-efficient and confirms a suspected rearrangement, such as the translocation t(1;9)(p36;q13) in a case of follicular lymphoma with an

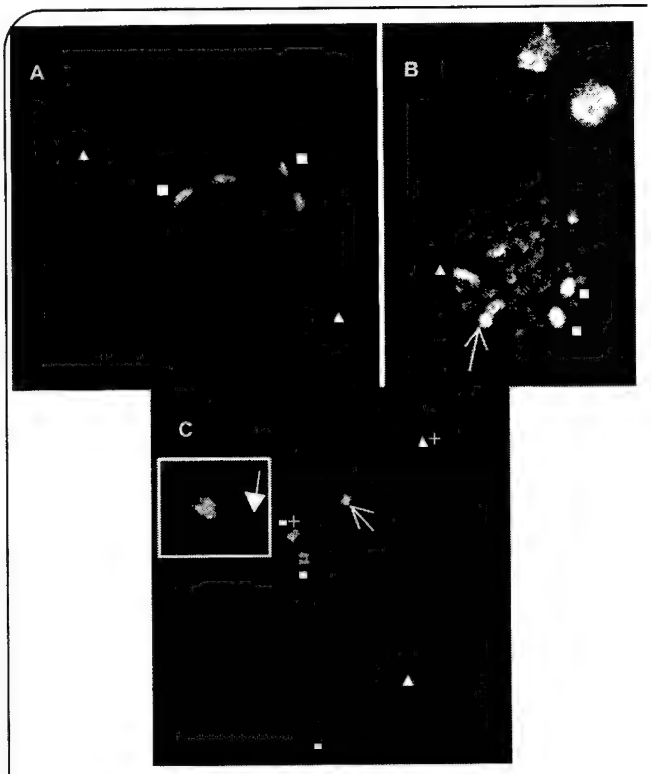

Figure 4. Whole chromosome painting (WCP) probes in the analysis of metaphase cells: (A) Dual-color hybridization delineating normal chromosomes 1 (square) and 8 (triangle) in a normal human metaphase spread; **(B)** Dual-color hybridization of WCP probes for chromosome 1 (square) and chromosome 9 (triangle) delineates normal and translocation chromosomes (arrow) in a leukemia case carrying a t(8;14) and a t(1;9). (Image courtesy of Dr. I Wlodarska, Leuven, Belgium); **(C)** Structural alterations in a thyroid cancer cell line lead to rearrangements of chromosomes 3 (square) and 17 (triangle). A dual-color hybridization reveals normal homologues (a plus mark next to the square or triangle), derivatives of either chromosomes 3 or 17 (square or triangle respectively) and a marker chromosome (solid arrowhead within insert).

additional t(14;18) shown in FIGURE 4B (the arrows point at rearranged chromosome). Using two WCP probes labeled with different reporter molecules also allows detection of translocations involving one or several stained chromosomes and any of the unstained chromosomes. FIGURE 4C illustrates this with an example from the analysis of a follicular thyroid cancer cell line. A chromosome 3-specifc WCP probe (triangle) was combined with a WCP probe for human chromosome 17 (square). An intact chromosome 3 and an intact chromosome 17 were detected. In addition, the metaphase spreads showed multiple rearrangements (open arrowhead) involving either chromosome 3 or chromosome 17 and unstained and as yet unidentified chromosomes. A marker chromosome (FIGURE 4C, insert) was found comprised of chromosome 3 and chromosome 17 material in addition to a small amount of DNA from an as yet unidentified chromosome. The unidentified material at one end of this marker chromosome was visualized by DAPI which counterstains all DNA (FIGURE 4C, solid arrowhead within insert). Chromosome painting is the method of choice to define larger breakpoint intervals in translocations like the ones shown here. Further detailed mapping of the breakpoints can then be performed by 'chromosome walking' using LSPs [12,13,46–48] or sets of probes spread along the target chromosomes in narrow distances [32,52,53].

SKY & M-FISH analyses

Complex rearrangements in tumor cells are mapped rapidly by using tumor metaphase cell preparations and combining a larger number of WCP probes. If the unambiguous classification of all chromosomes in a metaphase spread is required, the user can choose between SKY or filter-based M-FISH. The SKY approach is able to resolve fluorescence spectra with a resolution of about 10 nm, thus it records the equivalent of 30–40 distinctly different spectral images. This takes more time than recording 5–7 images with an M–FISH system and the user may want to weigh the advantages of high resolution against those of a higher throughput analysis.

FIGURE 5 illustrates the SKY analysis of a metaphase spread prepared from the human prostate cancer cell line TSUPR1. Images were acquired with an SD200 SpectraCube^{TM} Spectral Imaging system (ASI, Inc., Carlsbad, CA). The spectral imaging system attached to a Nikon E600 microscope consisted of an optical head (Sagnac interferometer) coupled to a multi-line CCD camera (Hamamatsu). The image data were stored in a Pentium 586/300 MHz computer and analyzed by proprietary software. The multiple band pass filter set used for fluorochrome excitation was custom-designed (SKY-1, ChromaTechnology, Brattleboro, VT) to provide broad emission bands (giving a fractional spectral reading from ~ 450 nm to ~ 850 nm). Using a Xenon light source, the spectral image was generated by acquiring 80–130 interferometric frames per object. The time needed to acquire the image was less than 3 min.

The DAPI image (FIGURE 5A) helped to identify chromosomes and chromosomal breakpoint regions. Following chromosome classification based on the full 450–850nm fluorescence spectrum, individual chromosomes were assigned 'classification colors' which linked the fluorescence spectra to chromosome-specific WCP probe mixtures (FIGURE 5A) [3,37]. SKY analysis of TSUPR1 metaphases revealed a number of previously unknown translocations (t(2;8),t(3;19),t(6;7), t(6;15),t(15;18)) in the presence of several marker chromosomes (FIGURE 5D). FIGURE 5A shows the inverted image of the chromosome spread acquired through the DAPI filter and FIGURE 5B shows the WCP probe fluorescence along the same chromosomes as an RGB pseudo-color image (presented in grayscale). Based on the measurement of the complete spectrum for each point in this metaphase image, a spectral classification algorithm allowed the assignment of a defined pseudo-color to all points in the image that displayed the same spectrum. A karyotype table was obtained (FIGURE 5C) showing 84 chromosomes with six of them classified as marker chromosomes. The karyotype table (FIGURE 5D) shows the chromosomes in classification colors to the left and normal ideograms to the right. Clearly, translocations in this cell prostate cancer cell line involved the chromosomes 2, 3, 4, 5, 7, 8, 11, 15, 18 and 19.

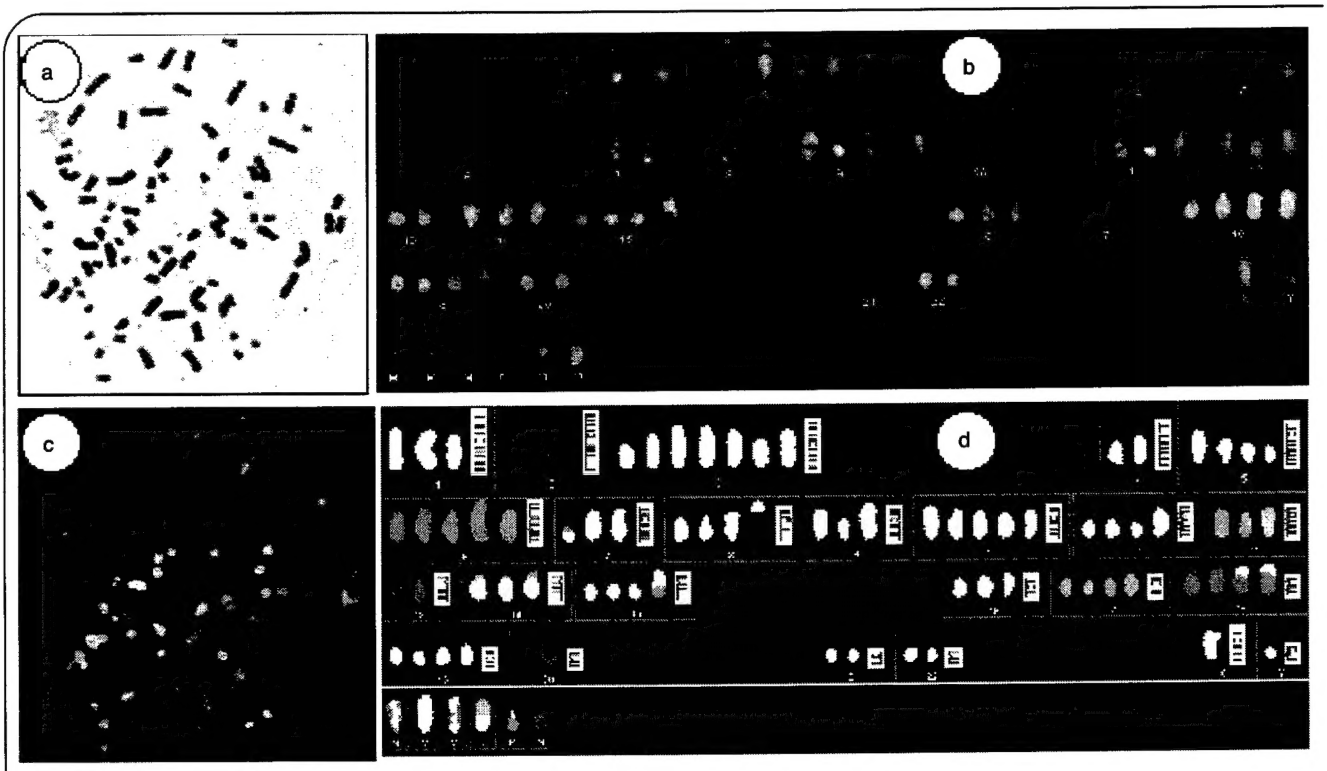

Figure 5. Spectral karyotyping (SKY) analysis of a tumor cell metaphase spread. SKY analyses of metaphase spreads from the human prostate cancer cell line TSUPR1: **(a)** Inverted DAPI image; **(b)** RGB pseudo-color image rendered in grayscale; **(c)** Karyotype table in grayscale; **(d)** Spectral karyotype showing chromosomes after assignment of classification colors to the left and normal idiograms to the right rendered in grayscale. Figures reproduced with kind permission of Springer Verlag [35].

The application of M-FISH is illustrated by the example shown in FIGURE 6. Metaphase spreads from a primary culture of cells from a female acral lentiginous melanoma (ALM) patient were hybridized with SpectraVysion probe mixture (Vysis) and analyzed using an M-FISH microscope system. The M-FISH images reveal several numerical and structural abnormalities summarized as karyotype of 44 (X0), t(8;1), t(6;15), t(3;22), del (2q), +7, -X, -10.

Both techniques, SKY as well as M-FISH, have detection limits in the megabasepair range [54]. The most common problem with the detection of small chromosomal fragments in complex translocations is the spatial overlap of combinatorially labeled probes. As Lee *et al.* [54] pointed out, the juxtaposition of material from nonhomologous chromosomes frequently results in overlapping fluorescence at the interface of the translocated segments, a phenomenon also referred to as 'flaring' [55]. In most cases, the negative impact of flaring and chromosome misclassification can be addressed by subsequent hybridization using chromosome-specific WCP probes [54].

Conclusion & expert opinion

Intensive biomedical research in the last two decades has increased our understanding of genetic changes underlying tumorigenesis and progression to a more malignant tumor phenotype. The International Human Genome Project and its many associated efforts have generated resources and tools that now enable researchers to identify genetic changes at the level of single cells.

Playing a pivotal role in the genetic analysis of single cells, FISH has frequently helped to unravel the complex changes accompanying tumor invasion and metastasis. FISH owes its wide acceptance to the fact that it has become a simple procedure bringing together two main components: cells and tissue specimens (provided by the researcher or clinician) and nonisotopically-labeled probes, many of which are now commercially available. The near future is likely to see a significant increase in the number of probes cleared by the US FDA for *in vitro* diagnostic use as well as ready-to-use reagents pushing the limits of detection to smaller targets and, at the same time, increase the number of targets that can be studied simultaneously. Instrument prices are unlikely to change but increased automation and knowledge-based information systems will facilitate the molecular cytogenetic analyses and reduce costs.

The FISH-based assays will be rapid, inexpensive and require only a small number of cells, thus providing an affordable diagnostic service to the large community of cancer patients in the USA and elsewhere. Information gained by these measurements can be utilized to enhance prognostication, therapeutic decisions and patient management or to measure the effects of drugs in laboratory as well as clinical studies. We steadily increase our knowledge about the relationship between genetic alterations and the course of the disease. Determining genetic changes beside the status of biochemical and histopathological markers will enable clinicians to provide patients with a more individualized therapy.

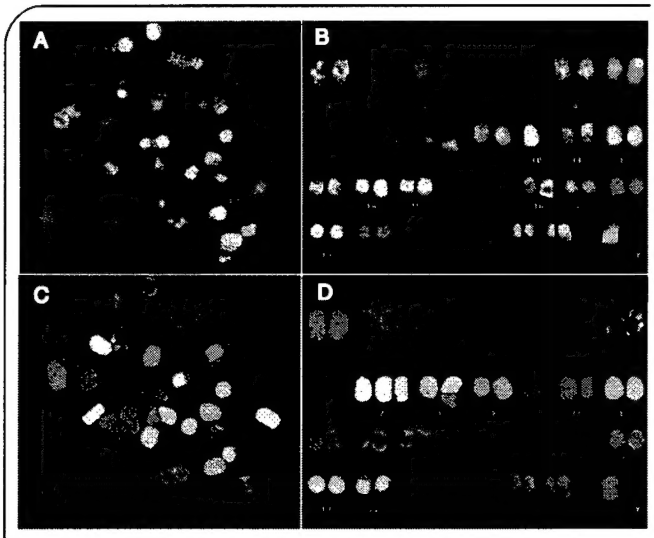

Figure 6. M-FISH analysis of human metaphase spreads reveals structural alterations in a case of acral lentiginous melanoma (ALM). Metaphase spreads from a primary culture of cells from a female ALM patient were hybridized with SpectraVysion probe mixture (Vysis, Inc.) and analyzed using an M-FISH microscope system. The M-FISH images reveal several abnormalities summarized as karyotype of 44 (X0), t(8;1), t(6;15), t(3;22), del (2q), +7, -X, -10. **(A)** RGB colored image of the metaphase spread rendered in grayscale; **(B)** the karyotype table in in grayscale; **(C)** the metaphase image after assignment of classification colors rendered in grayscale; **(D)** the karyotype table in classification colors rendered in grayscale

Five-year view

Rapid progress of the Human Genome Project and the completion of a draft sequence of the human genome have provided a course for even more comprehensive genetic analyses in the future. As key events and changes during the progression of normal cells to malignant tumors are deciphered, FISH probes and hybridization protocols will be developed to visualize these changes in individual cells. To the clinician, this will provide important information complementing histopathological analyses and thus may guide individualized therapeutic decisions.

While multicolor FISH techniques are poised to help decipher the complex changes underlying cancer, the early detection of tumor cells or minimal residual disease as well as identification of individuals at elevated risk faces several challenges. First, FISH technology must go beyond a simple detection of the somewhat static cytogenetic changes and address the relative levels of expression of genes involved in the tumor phenotype. A typical example is the overexpression of the erbB-2 protein, the product of the Her-2/neu proto-oncogene, in various forms of cancer. There is growing evidence that gene amplification in addition to overexpression is an important prognostic factor in breast cancer and future FISH techniques should be able to render information about the levels of both, amplification as well as gene expression.

Other concerns are directed towards the amount of material needed to perform a comprehensive genetic analysis of tumor cells by FISH. The successful analysis of single blastomeres biopsied from preimplantation human embryos [47,48,56] has opened up new avenues for the analysis of small samples. Future technology developments will be geared towards the generation of a large amount of cytogenetic information from a small number of cells.

As the main thrust of research in the postgenome era shifts to proteomics, FISH techniques will need to change and accommodate immunocytochemical techniques, or minimally, be able to combine nucleic acid analyses with protein quantitation and phenotypic characterization using tumor marker-specific antibodies. Second, to conduct population studies, FISH technology needs to become standardized and automated. Present hands-on procedures must be modified to allow the processing of hundreds or even thousands of specimens within reasonable timeframes. This also calls for knowledge-based, automated analysis systems able to gather and categorize information at very high speed. While most of the necessary technology already exists, it will take continued funding and engineering ingenuity to bring the pieces together. Finally, the power of FISH-based techniques for patient diagnosis is now beginning to be harnessed. Many health professionals outside the field of molecular genetics must be trained to correctly interpret FISH data. Computer professionals, who will design and implement future software and primary care professionals, who work directly with the patients, must be trained to understand FISH data. This training may turn out to be as challenging to achieve as the technical horizons we have mapped out above.

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Key issues

- FISH is non-isotopic method to localize nucleic acids, such as DNA and RNA.
- Increased application of FISH assays in the clinical practice will require thorough training of healthcare professionals.
- Probes used for FISH are labeled with nonradioactive haptens, such as biotin, digoxigenin or fluorochromes. They are stable for several years and can be disposed of without hazards. Several FISH probes labeled with different reporter molecules can be combined for multilocus analyses. Many FISH probes are now commercially available.
- FISH assays can be set up to investigate specific alterations which are typical for a particular tumor or they can be designed to screen the entire genome for alterations.
- FISH is sensitive and rapid.
- FISH results support the molecular staging of tumors, thus facilitate prognostication and individualized treatment regimens.
- FISH assays detect numerical as well as structural alterations in a wide variety of cells and tissues and, as our understanding of the cytogenetic changes underlying tumorigenesis, invasion and metastasis increases, will evolve to specifically address the etiology of each disease.
- To be able to handle a significant larger number of clinical samples, FISH techniques need to be automated. There is also a growing need to design digital imaging systems and expert computer systems for FISH analyses in the clinical laboratory.

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